

Destabilization of Steroid Receptors by Heat Shock Protein 90-binding Drugs: A Ligand-independent Approach to Hormonal Therapy of Breast Cancer¹

Rochelle Bagatell,² Omar Khan,
Gillian Paine-Murrieta, Charles W. Taylor,
Shiro Akinaga, and Luke Whitesell

Department of Pediatrics, Steele Memorial Children's Research Center [R. B., O. K., L. W.], and Arizona Cancer Center [G. P.-M., C. W. T.], University of Arizona, Tucson, Arizona 85724, and Pharmaceutical Research Institute, Kyowa Hakko Kogyo Co., Ltd., Shizuoka 100, Japan [S. A.]

ABSTRACT

Steroid hormone receptors have become an important target in the management of breast cancers. Despite a good initial response rate, however, most tumors become refractory to current hormonal therapies within a year of starting treatment. To address this problem, we evaluated the effects of agents that bind the molecular chaperone heat shock protein 90 (Hsp90) on estrogen receptor function in breast cancer. Unstimulated estrogen and progesterone receptors exist as multimolecular complexes consisting of the hormone-binding protein itself and several essential molecular chaperones including Hsp90. We found that interaction of the Hsp90-binding drugs geldanamycin and radicicol with the chaperone destabilizes these hormone receptors in a ligand-independent manner, leading to profound and prolonged depletion of their levels in breast cancer cells cultured *in vitro*. Consistent with these findings, *in vivo* administration of the geldanamycin derivative 17-allylaminogeldanamycin (17AAG; NSC330507) to estrogen-supplemented, tumor-bearing SCID mice resulted in marked depletion of progesterone receptor levels in both uterus and tumor. Drug administration also delayed the growth of established, hormone-responsive MCF-7 and T47D human tumor xenografts for up to 3 weeks after the initiation of therapy. We conclude that in light of their novel mechanism of anti-hormone action, consideration should be given to examining the activity of 17AAG and other Hsp90-binding agents in patients with refractory breast cancer in future clinical

trials, either alone or in combination with conventional hormone antagonists.

INTRODUCTION

Over the past two decades, steroid receptors have become increasingly prominent targets for the prevention and treatment of hormone-dependent cancers. In particular, anti-estrogens such as tamoxifen are now a cornerstone in the management of ER³-positive tumors (1). Unfortunately, most metastatic breast cancers eventually become refractory to hormonal therapy, often through acquired alterations in the structure, expression, or activity of the estrogen binding subunit of the receptor itself (2, 3). Extensive efforts have been directed at the development of selective estrogen receptor modulators that can repress estrogen-induced transcriptional activation of proliferation-associated genes while retaining agonist activity in relation to other estrogen-responsive target genes. Although they provide the potential for substantial improvements in selectivity and the avoidance of undesirable side effects, selective estrogen receptor modulators remain dependent on the high affinity interaction of antagonist with the hormone binding subunit of the receptor (4, 5). Because the frequent emergence of resistance to currently available antagonists limits their overall effectiveness as anticancer agents, the identification of compounds that inhibit receptor function in an alternative, ligand-independent manner could prove very useful.

Unstimulated steroid hormone receptors exist as multimolecular complexes containing the hormone binding protein itself and a number of essential chaperone proteins including Hsp90 (6). Inhibition of Hsp90 function prevents the assembly of the complexes required to maintain hormone receptors in their mature, ligand-binding configuration (7). In the past 5 years, the antitumor antibiotics GA and RD have become recognized as selective HBAs with a novel ability to alter the activity of many of the receptors, kinases, and transcription factors involved in cancer-associated signaling pathways. These drugs have now been shown by crystallographic (8, 9) and biochemical (10, 11) analyses to bind as nucleotide mimetics to an NH₂-terminal ATP/ADP-binding domain within Hsp90, locking the chaperone in its ADP-bound conformation and compromising its function. Although GA and RD are active *in vitro*, derivatives of these parent compounds including 17AAG and the RD oxime derivative KF58333 have been developed which show greater *in vivo* efficacy at well-tolerated doses (12, 13). Fig. 1 provides a

Received 2/15/01; revised 4/3/01; accepted 4/4/01.

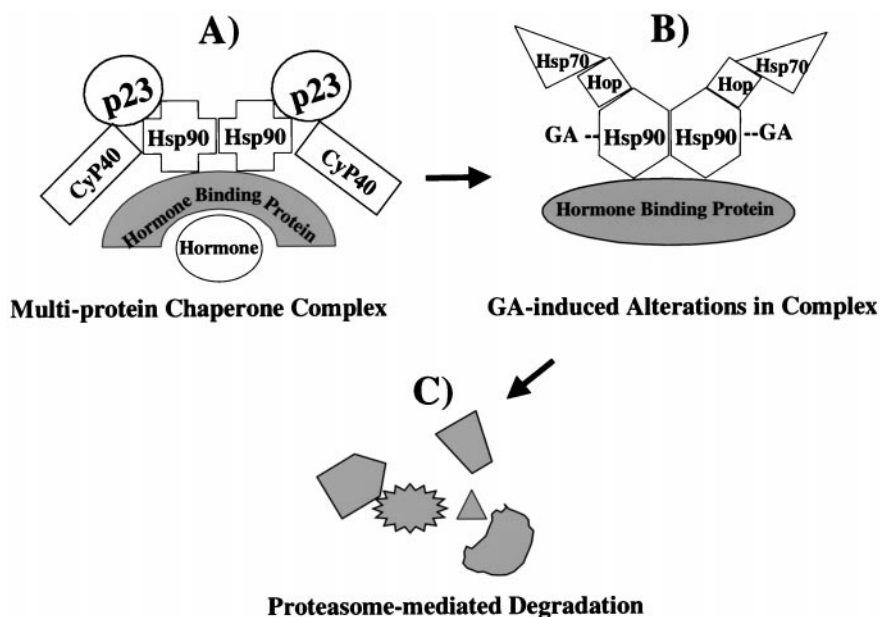
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹This work was supported in part by NIH Grants CA69537 and CA09213 and funds from the Caitlin Robb Foundation.

²To whom requests for reprints should be addressed, at Pediatric Hematology/Oncology, Steele Memorial Children's Research Center, University of Arizona, 1501 North Campbell Avenue, Tucson, AZ 85724. Phone: (520) 626-4851; Fax: (520) 626-4220; E-mail: bagatell@peds.arizona.edu.

³The abbreviations used are: ER, estrogen receptor; Hsp, heat shock protein; GA, geldanamycin; RD, radicicol; HBA, Hsp90-binding agent; 17AAG, 17-allylaminogeldanamycin; PR, progesterone receptor; GR, glucocorticoid receptor; SCID, severe combined immunodeficient.

Fig. 1 Schematic representation of steroid hormone receptor complexes and the effects of HBAs on receptor structure and stability. In **A**, the physical association of several molecular chaperones stabilizes the hormone-binding subunit of the receptor and maintains it in a conformation capable of high-affinity hormone binding. *Cyp40*, cytochrome P-450. In **B**, the interaction of GA with Hsp90 alters its conformation and the composition of chaperone complexes found in association with the hormone-binding protein. In the case of GR, PR, and androgen receptor but not ER, these changes lead to a rapid loss of the high-affinity hormone binding conformation of the receptor. In **C**, drug-induced alterations in chaperone function led to the depletion of cellular hormone-binding protein levels via proteasome-mediated degradation.



simplified model summarizing previous work done by us and by others to elucidate the effects of drug interaction with Hsp90 on the structure and function of hormone receptor complexes. In their native state, steroid receptors consist of the relevant hormone binding protein itself, an Hsp90 dimer, and several co-chaperones, such as p23, and immunophilins, such as Cyp40 (Fig. 1A). When GA and the other known HBAs bind Hsp90, as illustrated in Fig. 1B, the conformation and function of Hsp90 are altered. The physical association of p23 and Cyp40 is no longer detected, whereas that of other chaperones, such as Hsp70 and Hop, becomes apparent (7). In the case of the PR, androgen receptor, and GR, drug-mediated disruption of Hsp90 function results in a rapid loss of high-affinity hormone binding activity (14, 15). This effect has been demonstrated both *in vitro* using biochemical receptor reconstitution assays and after drug treatment of whole cells. Loss of hormone binding is observed within 15 minutes of drug exposure. In addition, HBA treatment of cells also leads to increased proteasome-mediated degradation of the hormone binding proteins and a decline in their total cellular levels (Fig. 1C; Ref. 14). In contrast, we now show that GA does not rapidly disrupt high-affinity hormone binding by the ERs expressed in breast cancer cells, but it does cause receptor destabilization and a marked decline in cellular ER levels. Depletion of receptor levels can be achieved in culture and after treatment of tumor-bearing mice with HBAs at systemically tolerated doses, suggesting that it may be possible to exploit their novel mechanism of receptor inhibition therapeutically either alone or in combination with conventional antagonists.

MATERIALS AND METHODS

Cells and Reagents. MCF-7 and T47D cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured at 37°C under 6% CO₂ in air using RPMI 1640 (Life Technologies, Inc.) supplemented with 10%

fetal bovine serum (Irvine Scientific, Santa Ana, CA), 10 mM HEPES (Life Technologies, Inc.), and 2 mM L-glutamine (Life Technologies, Inc.). Cells were confirmed negative for *Mycoplasma* contamination by ELISA, and all experiments were performed within 10 serial passages. Viable cell numbers were quantitated by counting cells suspended in 0.2% trypan blue in a hemacytometer. RD and its derivative KF58333 were supplied by the Pharmaceutical Research Institute. GA and its derivative 17AAG were provided by the Developmental Therapeutics Program of the National Cancer Institute.

Metabolic Labeling and Receptor Immunoprecipitation. To examine the effects of drug treatment on receptor turnover, replicate dishes of MCF-7 cells were rinsed with methionine/cysteine-free DMEM supplemented with 10% dialyzed fetal bovine serum and incubated at 37°C for 1 h in the presence of GA (1.7 μM) or an equal volume of DMSO diluent (Sigma Chemical Co., St. Louis, MO). Cells were then labeled in the same medium supplemented with [³⁵S]methionine/[³⁵S]cysteine (Translabel, 10.5 mCi/ml; ICN, Costa Mesa, CA) to yield 100 μCi/ml. Incubation was continued for an additional hour, and then cells were washed and refed with methionine-containing medium in the continued presence of GA or DMSO. Cell lysates were prepared at 0, 2, 4, and 8 h after washout. Immunoprecipitation was performed from metabolically labeled cell lysates using the monoclonal anti-ER antibody H-151 (Stressgen, Victoria, British Columbia, Canada) as described previously (14).

Immunoblotting. To evaluate cellular levels of ER and Hsp72, replicate dishes of MCF-7 and/or T47D cells were treated with drug or diluent and lysed in nonionic detergent buffer. Immunoblotting was performed using 100 μg of total protein/sample as described previously (16). Primary antibodies used included anti-Hsp72 (C-92) obtained from Stressgen and anti-PR (MC243) and anti-ER (ER17), both generously provided by David Toft (Mayo Clinic, Rochester, MN). Peroxidase-

conjugated secondary antibodies, chemiluminescent substrate, and exposure to Kodak XAR-5 film were used for detection. Multiple exposure times were evaluated for each blot to ensure that the band intensities observed were within the dynamic response range of the film.

Quantitation of Estrogen Binding. MCF-7 cells were established in 24-well plates using phenol red-free RPMI 1640 supplemented with 10% charcoal-stripped fetal bovine serum. Cells were incubated for 72 h and then exposed for 1 h to 1 ml of medium containing tritiated 17β -estradiol (1 nM, 0.08 μ Ci/ml; NEN Life Science Products, Boston, MA) with or without 200-fold excess nonradioactive diethylstilbestrol (Sigma Chemical Co.). Wells were washed three times and extracted with ethanol for 20 min. Aliquots of the ethanolic solution were transferred to scintillation vials and counted. For each drug treatment condition, the protein content of replicate wells was determined by lysing the cells in nonionic detergent buffer and assaying for protein concentration using bicinchoninic acid reagent (BCA; Pierce, Rockford, IL). Specific hormone binding was calculated as the cpm per μ g of cellular protein bound in the absence of excess nonradioactive steroid less the cpm/ μ g protein bound in its presence.

In Vivo Studies. Prior to all experimentation, SCID mice (Arizona Cancer Center breeding colony; C.B.-17/IcrACCscid) were implanted s.c. with 17β -estradiol pellets (0.25 mg/21 day release; Innovative Research of America, Sarasota, FL). Non-tumor-bearing mice were treated with 50 mg/kg 17AAG formulated in DMSO and injected i.p. daily for 2 days. Control animal were treated with an equal volume of DMSO according to the same schedule. Animals were sacrificed 18 h after the last injection, and tissues were harvested. Snap-frozen tissues were pulverized on dry ice, and cytosolic extracts were prepared in hypotonic buffer (pH 8.2) containing 10 mM HEPES, 1 mM EDTA, and 10 mM sodium molybdate. For quality control purposes, lysates were routinely fractionated by 7.5% SDS-PAGE, followed by Coomassie staining, to evaluate the overall pattern of bands observed. Only those samples found to be free from extensive proteolysis or heavy contamination by serum albumin were evaluated by subsequent SDS-PAGE, followed by immunoblotting using anti-Hsp72 and anti-PR primary antibodies. To assess drug effects on hormone receptor levels within human tumor xenografts, estrogen-supplemented SCID mice were inoculated s.c. in the right lower flank with T47D breast cancer cells (10^7 cells) suspended in 200 μ l of a 1:1 mixture of saline and Matrigel (Collaborative Biomedical Products, Bedford, MA). After the formation of palpable tumors, mice received i.p. injections of 17AAG daily for 2 consecutive days. Control mice received i.p. injections of an equal volume of DMSO. Mice were sacrificed, and tumors and organs were resected 18 h after final drug injection. Tissue extracts were prepared, evaluated, and analyzed by immunoblotting as described above. To assess drug effects on tumor progression, estrogen-supplemented mice were inoculated s.c. with T47D cells suspended in saline/Matrigel as described above. Once palpable tumors were apparent (8–10 days after injection), mice were treated with 17AAG daily for 5 days in an effort to simulate the regimen currently being used in clinical trials in humans. Control animals were treated with an equal volume of DMSO according to the same schedule. Serial tumor measure-

ments were performed in two dimensions with a caliper tumorimeter, and volume was estimated using the formula: length \times width²/2 (17). All experiments involving mice were carried out under protocols approved by the University of Arizona Institutional Animal Care and Use Committee.

RESULTS

HBAs Decrease ER Levels and Hormone Binding in Breast Cancer Cells. MCF-7 breast cancer cells were used to evaluate the effects of GA, the prototypic HBA, on ER stability and function in whole cells. A pulse-chase experiment performed after a 1-h labeling period demonstrated that although the intensities of the bands corresponding to the ER are equivalent at the start of the chase period, a more rapid decline in ER signal is observed over time in lysates prepared from GA-treated cells than in lysates from control cells (Fig. 2A). Synthesis of ER in treated and untreated cells thus appears to be approximately equivalent, whereas turnover of ER is clearly increased in the GA-treated cells. The identities of the slower migrating bands that coprecipitate with the receptor in this experiment are not certain, but based on their apparent molecular size and their induction by GA, they are most likely Hsp70 isoforms that as chaperones have a tendency to bind Sepharose beads nonspecifically. To determine whether accelerated ER turnover leads to alterations in total cellular levels of the receptor after GA exposure, immunoblotting of lysates from treated and untreated cells was performed. The level of ER detected in lysate from MCF-7 cells maintained for 24 h in GA-containing medium is markedly decreased compared with that seen in lysate from cells treated with drug vehicle alone (Fig. 2B). A comparable decrease in ER protein level is also seen in cells that were exposed to the HBAs for 1 h and then washed and cultured in drug-free medium for 23 h prior to lysis and immunoblotting. This finding indicates that a brief drug exposure can still result in sustained depletion of receptor. To assess the consequences of GA treatment on receptor function, we used a whole cell radioligand binding assay. Consistent with the depletion of ER protein levels seen in Fig. 2B, specific binding of 17β -estradiol is markedly decreased by GA exposure but only at later time points (Fig. 2C). This delay in loss of binding is consistent with depletion of hormone-binding protein rather than direct disruption of binding activity *per se*. It stands in marked contrast to our previously reported findings with the GR, where >80% of dexamethasone binding was lost within 10 min of drug application (14).

To examine whether the observed effects on ER level and function are unique to GA or can be attributed more generally to disruption of Hsp90 function, we used the chemically distinct compound RD which, like GA, has been shown by crystallographic and biochemical analyses to bind to Hsp90. Fig. 3 shows that treatment of MCF-7 cells with RD causes a dose-dependent decrease in the level of ER. Previous work in our laboratory has demonstrated that HBAs exposure results in a heat shock factor 1 (HSF1)-dependent increase in cellular Hsp levels in mammalian cells, and that changes in levels of these proteins can serve as a positive marker of HBA action (18). As expected, after treatment with RD, the decrease in ER protein is accompanied by a marked increase in Hsp72, a highly inducible isoform of Hsp70. These data demonstrate that the decline in

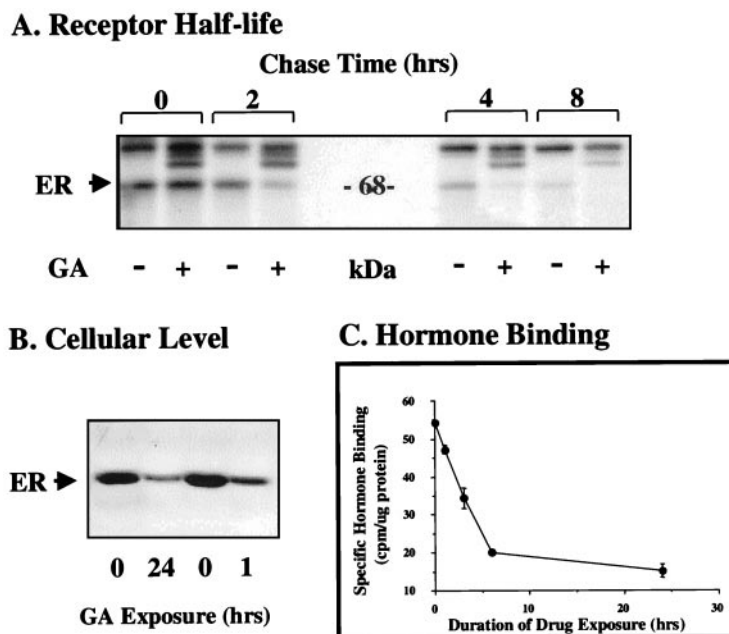


Fig. 2 GA destabilizes the ER and inhibits specific hormone binding by MCF-7 cells. In **A**, cells were treated with GA (1 $\mu\text{g/ml}$) or an equal volume of DMSO for 1 h, followed by metabolic labeling with [^{35}S]methionine/[^{35}S]cysteine for 1 h. Cells were then washed, refed with methionine/cysteine-containing medium in the continued presence of GA or DMSO as indicated, and lysed after the chase periods indicated. Immunoprecipitation of ER was performed from equal amounts of trichloroacetic acid-precipitable radioactivity, and precipitates were fractionated by 10% SDS-PAGE. Labeled proteins were visualized by autoradiography. *Arrow*, migration position of the ER. In **B**, cells were exposed to GA (1 $\mu\text{g/ml}$) or control solvent either continuously for 24 h or for 1 h, followed by washout and continued culture for 23 h. Cells were then lysed, and the level of ER protein in 100 μg of total cellular protein was analyzed by immunoblotting. In **C**, cells growing under hormone-depleted conditions were assayed for their ability to bind ^{125}I -estradiol. Replicate wells were incubated with radiolabeled estradiol in the presence or absence of a 200-fold molar excess of diethylstilbestrol. After extensive washing, wells were extracted with ethanol, and the radioactivity in each extract was quantitated by liquid scintillation counting. The protein content of replicate wells was determined by BCA assay for each treatment condition. Specific hormone binding is depicted as the cpm/ μg of total cellular protein bound in the absence of nonradioactive steroid less the mean cpm/ μg bound in its presence. Each point represents the mean of triplicate determinations; *bars*, SD.

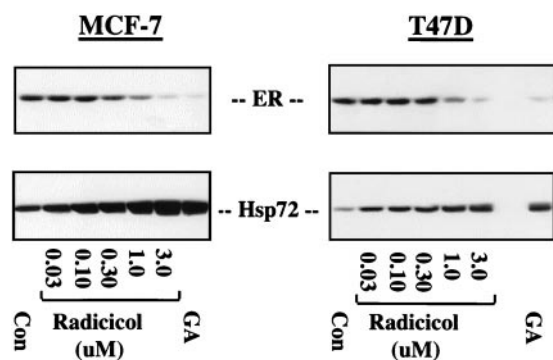


Fig. 3 Concentration-dependent depletion of cellular ER levels by RD. MCF-7 (*left*) and T47D (*right*) breast cancer cells were incubated in the indicated concentrations of RD overnight, followed by lysis and evaluation of ER and Hsp72 levels by immunoblot. *Con*, lysate prepared from cells exposed to an equal volume of DMSO. *GA*, lysate from cells exposed to GA (1.7 μM) for the same overnight incubation period.

receptor levels observed are not simply attributable to drug-induced cytotoxicity/cell death because the cells are still capable of mounting a stress response. As an internal control to allow comparison with the results in Fig. 2, cells were also treated

with GA (1.7 μM), and the expected pattern of increase in Hsp72 and decrease in ER was observed. To determine whether these results are specific to the MCF-7 cell line, experiments were repeated using the T47D breast cancer cell line that expresses both ER and PR. Again, a dose-dependent decrease in ER level and an increase in Hsp72 are seen after exposure to RD and GA.

HBA's Decrease Cellular PR Levels. In preparation for whole animal studies, we examined the effects of HBA with activity *in vivo* on PR levels in T47D cells. It was important to validate the PR as a reliable end point for drug action in these experiments because of the difficulties associated with measuring ER levels in tissues from estrogen-supplemented mice (see "Discussion"). After overnight treatment of cells with KF58333, an oxime derivative of RD that is currently in pre-clinical development, lysates were analyzed by immunoblot. Fig. 4A shows the expected induction of Hsp72 at drug concentrations $>0.01 \mu\text{M}$ and a marked decrease in the level of ER, as well as a decrease in the A and B isoforms of the PR. Because we were concerned that HBA effects on receptor stability could be altered by the presence of ligand, we also examined receptor levels in cells cultured in hormone-supplemented medium. As shown in Fig. 4B (*upper panel*), supplementation with either $^{17}\beta$ -estradiol or progesterone had little effect on the ability of KF58333 to deplete cellular PR levels. To confirm that this

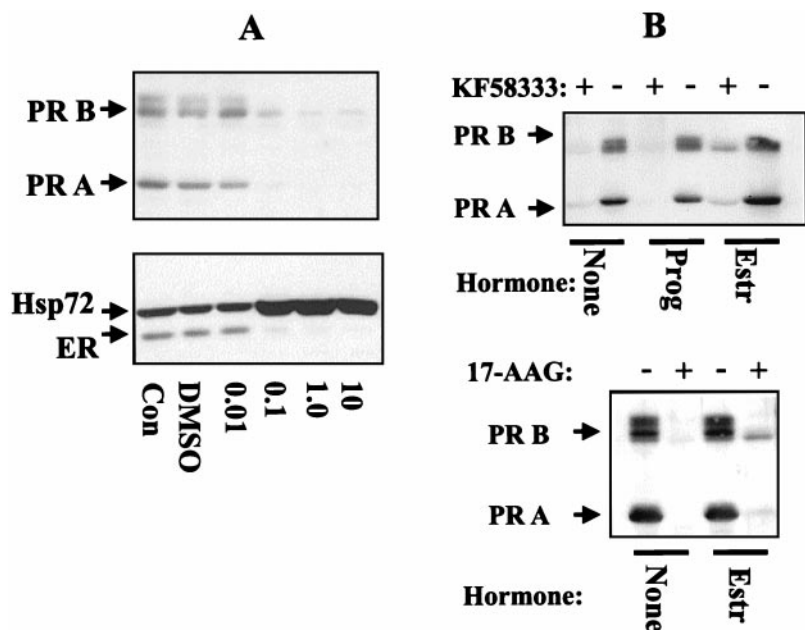


Fig. 4 HBA exposure depletes cellular hormone receptor levels, even in the presence of hormone supplementation. In *A*, replicate dishes of T47D cells were incubated overnight with the indicated concentrations of KF58333 (μM), an oxime derivative of RD with activity in mice. Additional dishes were incubated with either no drug (*Con*) or an equal volume of DMSO solvent. Cells were then lysed, and equal amounts of total protein were analyzed by immunoblot for the levels of PR (A and B isoforms), ER, and Hsp72, as indicated by the *arrows*. In *B*, cells were incubated overnight with either KF58333 (1 μM , + lanes, *upper panel*) or 17AAG (1 μM , + lanes, *lower panel*) as indicated. An equal volume of DMSO solvent was added to replicate dishes to generate lysates for the control (-) lanes. Supplemental hormone was added to some dishes 1 h prior to drug addition as follows: 17 β -estradiol, 10 nM (*Estr lanes*); progesterone, 10 nM (*Prog lanes*); and an equal volume of ethanol vehicle (*None lanes*). Lysates were prepared and analyzed by immunoblot for PR level as in *A*.

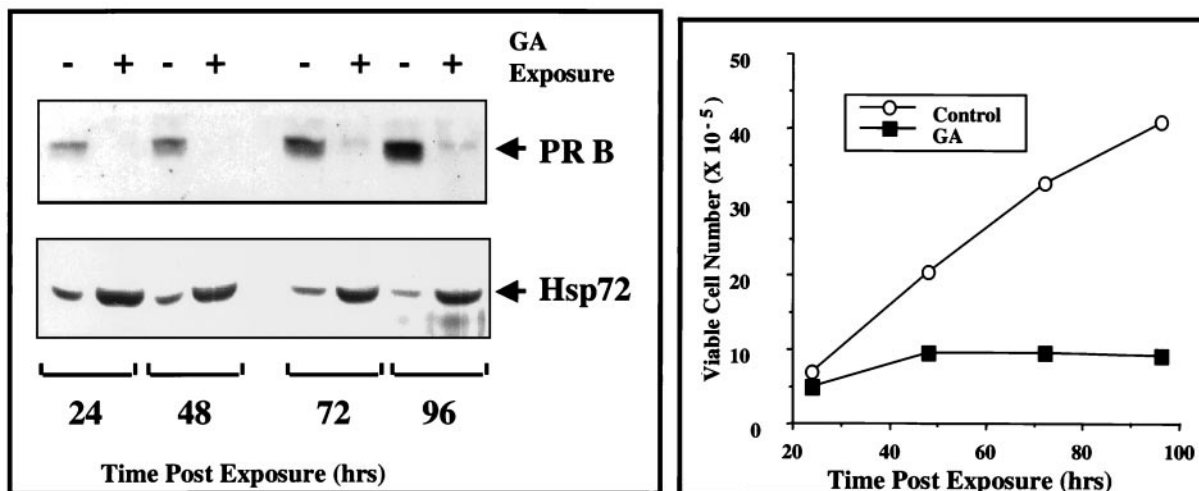


Fig. 5 Brief exposure to GA results in prolonged depletion of PR *in vitro*. Replicate dishes of T47D cells were incubated with GA (1 μM) or an equal volume of DMSO for 1 h, followed by washout. Control and drug-treated dishes were harvested by trypsinization at the indicated intervals after drug exposure. Aliquots were analyzed to determine viable cell number by trypan blue counting (*right panel*). The remainder of each sample was lysed to determine its relative level of PRB and Hsp72 by immunoblot once all of the samples had been collected (*left panel*).

effect is not specific to a single HBA, we also treated estrogen-supplemented T47D cells with 17AAG, a derivative of GA currently in Phase I clinical trial. The decrease in PR observed after exposure of cells to 17AAG (Fig. 4*B*, *lower panel*) is comparable with the change seen after treatment with KF58333 and only minimally altered by estrogen supplementation.

PR Depletion by HBAs Is Durable and Associated with a Decrease in Tumor Cell Growth. To simulate the type of bolus drug exposure that would be most relevant to clinical use, we exposed replicate cultures of T47D cells to GA for 1 h and then lysed cells at 24-h intervals after treatment. Brief exposure

to GA resulted in a surprisingly durable induction of Hsp72, accompanied by persistent depletion of PR for up to 96 h after initial drug pulse (Fig. 5, *left panel*). As might be expected, the data also demonstrate that basal PR levels gradually increase over time in control cells as they grow to confluency. To assess whether the prolonged decrease in hormone receptor level observed after HBA exposure was simply a reflection of cytotoxicity, aliquots of the T47D cells exposed to GA or to diluent for 1 h were also counted in trypan blue at 24, 48, 72, and 96 h after treatment. As shown in Fig. 5 (*right panel*), control cells show rapid growth as expected, whereas drug-treated cells remain

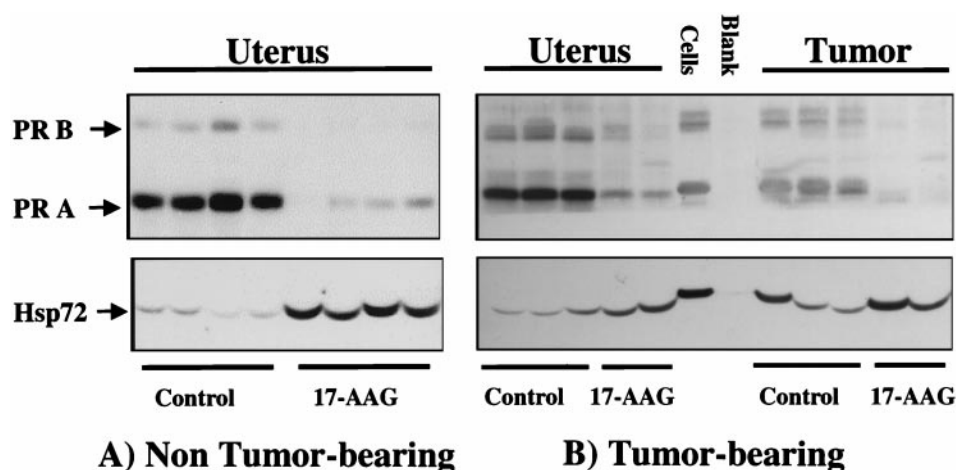


Fig. 6 Depletion of PR by 17AAG treatment of mice. Estrogen-supplemented SCID mice were injected i.p. with 17AAG or an equal volume of DMSO vehicle (50 μ l) daily for a total of two doses. They were euthanized 18 h after the last dose, and tissues were resected and snap frozen for determination of relative PR and Hsp72. In *A*, four non-tumor-bearing mice/group were treated with 17AAG 50 mg/kg/dose or DMSO, and uterus lysates were analyzed by immunoblot. In *B*, three mice bearing established T47D tumors/group were treated with 17AAG (75 mg/kg/dose). Uterus and tumor lysates were analyzed by immunoblot. As a positive control for the blotting procedure, lysate prepared from T47D cells in culture (*Cells lane*) was also analyzed.

viable but fail to proliferate for at least 4 days after drug exposure. This type of experiment was also performed with KF58333 and 17AAG, and similar results were observed (data not shown), leading us to conclude that the sustained depletion of cellular PR levels observed after HBA exposure is not simply attributable to cytotoxicity/cell death.

Systemic Administration of HBA Decreases PR Levels in Target Tissues *in Vivo*. Non-tumor-bearing, estrogen-supplemented SCID mice were treated with 17AAG and then sacrificed 18 h after the last dose. Immunoblotting of lysates derived from uterine tissue demonstrated a marked increase in Hsp72 levels in tissue from the treated animals compared with controls (Fig. 6A). Analysis of PR isoforms shows that drug administration led to a marked decrease in levels of PR A and PR B in all animals treated with drug, with almost undetectable levels of PR seen in lysate from one animal. We next used animals bearing human tumor xenografts to assess changes in PR after administration of 17AAG. Estrogen supplemented SCID mice were injected with T47D tumor cells s.c. After the formation of palpable tumors, they were treated with 17AAG (75 mg/kg immunoprecipitation) daily for 2 days and then euthanized 18 h later. Fig. 6B shows immunoblots for PR and Hsp72 of lysates prepared from the uterus (*left lanes*) and tumor (*right lanes*) of three control and two drug-treated mice. Consistent with results in non-tumor-bearing mice, levels of Hsp72 are generally higher in uterus and tumor from drug-treated animals compared with tissues from controls. There does appear to be greater variability in Hsp72 levels in lysates from these animals, perhaps because of the stress of bearing tumor burdens. Nonetheless, a consistent decrease in the level of PR is seen in the lanes containing lysate from the uterus and tumor of drug-treated animals. The lysate from the tumor and uterus of a third drug-treated mouse in this experiment was not blotted because of excessive proteolytic degradation of the sample revealed by Coomassie staining (data not shown). In several experiments of

similar design, lysates from a small but consistent minority of animals were also found to be degraded. Although this may have been a technical problem generated during preparation of lysates, the fact that degradation occurred only in tumor lysates from HBA-treated mice and never in lysates from uteri of control or drug-treated mice suggests that this finding may reflect the antitumor activity of the drug treatment itself.

Treatment with 17AAG Delays Growth of Human Tumor Xenografts. Given the demonstrated ability to modulate PR *in vivo* with administration of 17AAG, we next evaluated the effects of this drug on tumor growth. Estrogen-supplemented SCID mice bearing established tumors were treated with 17AAG, and tumor volumes were assessed using serial caliper measurements. Fig. 7 demonstrates that 17AAG treatment at systemically tolerated exposures significantly altered tumor growth in mice as determined by two-way ANOVA ($P < 0.0001$). Progression of T47D tumor growth is inhibited by HBA treatment for up to 3 weeks after a course of therapy.

DISCUSSION

The documented activity of hormonal therapies in the treatment and prevention of breast cancer has made the ER a well-validated therapeutic target in this common and often incurable malignancy. To date, hormonal therapies have been directed at blocking the ER (hormone antagonists such as tamoxifen) or reducing the synthesis of estrogens in postmenopausal women (aromatase inhibitors such as anastrozole). Although clinical trials have demonstrated a roughly 30% initial response rate (complete response + partial response) to such therapies in patients with advanced breast cancer patients (19), the responses are typically short-lived. A recent report found that median time to progression for postmenopausal women with advanced breast cancer treated with tamoxifen was 5.6 months (20). Patients whose tumors are ER⁺ and/or PR⁺ are

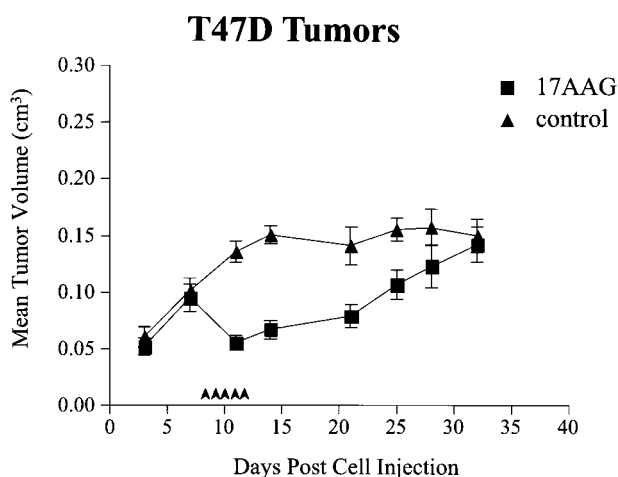


Fig. 7 Inhibition of tumor growth by 17AAG. Estrogen-supplemented SCID mice bearing established breast tumor xenografts were injected with 17AAG (50 mg/kg/dose i.p. daily for 5 days) or an equal volume of DMSO (50 μ l), and mean tumor volumes were measured serially. Each group consisted of 10 animals, and the means are depicted; bars, SE.

considered most likely to respond favorably to tamoxifen therapy, but investigators have shown that even in this group median time to progression was 7.8 months, and by 2 years from the time of initiation of tamoxifen therapy, >85% of patients had progressed (21). Clearly, alternative approaches to manipulate hormone signaling are needed if durable responses are to be achieved. To address this issue, we now report the feasibility of destabilizing steroid hormone receptors in breast cancer cells and human tumor xenografts using drugs that alter the function of the molecular chaperone, Hsp90 (Fig. 1).

Numerous studies have made clear that Hsp90 in conjunction with several other chaperones and cofactors modulates hormone binding and receptor stability but to different extents, depending on the specific steroid receptor and assay system being evaluated (22, 23). In the case of the ER, Hsp90 clearly forms complexes with the receptor, but its precise role in facilitating high-affinity hormone binding has remained controversial (24). Recently, the anticancer antibiotics GA and RD have been identified based on their ability to bind Hsp90 and alter its function (11, 25). Studies in our lab (7, 14) and those of several other investigators (reviewed in Refs. 26 and 27) have examined the molecular effects of these HBAs on steroid receptor structure, function, and stability. In this report, we show that unlike their effects on GR and PR, HBAs at low micromolar concentrations do not directly disrupt specific hormone binding by the ER but do destabilize the receptor, leading to its depletion in drug-treated cells (Figs. 2 and 3). On the basis of these findings in culture, we went on to examine the activity of HBAs in SCID mouse breast tumor xenografts models. To the best of our knowledge, this is the first published report demonstrating that depletion of a steroid hormone receptor can be achieved in tumor-bearing mice by using systemically tolerated doses of 17AAG (Fig. 6). It has been suggested recently that the novel "pure" antiestrogen ICI 182,780 causes increased degradation of ER (28). Unlike the ligand-independent HBA effect on ER,

however, ICI 182,780 mediated ER depletion is dependent upon binding of this ligand to the receptor. HBAs, then, appear to possess a novel mechanism of action and therefore could prove useful either alone or in combination with receptor antagonists and aromatase inhibitors to achieve more effective blockade/ablation of estrogen action.

Although far from completely understood, acquired resistance to antiestrogens appears to involve multiple molecular mechanisms including: alterations in the metabolism of the antiestrogen itself, mutation of the ER hormone binding protein, alterations in the cross-talk between relevant signal transduction pathways, and alterations in the balance of nuclear coactivators and corepressors (Ref. 29 and references therein). Given the ligand-independent nature of HBA action (Fig. 4), however, these mechanisms are unlikely to impair the ability of drugs such as 17AAG and KF58333 to destabilize receptors and disrupt hormone action. Over time, alterations in drug metabolism and/or disposition may affect the efficacy of HBAs in patients with breast cancer, but mutation of the primary target of HBAs would seem unlikely. Hsp90 is a highly conserved molecular chaperone that plays an important role in many essential cellular functions in addition to its involvement with steroid receptors. As a result, mutations that alter its ability to bind HBAs would probably be quite deleterious to cell survival. In support of this contention, mutation of Hsp90 has not been reported in any cancers, although overexpression of certain chaperones, especially Hsp70, has been documented (30).

Hsp90 knock-out is known to be lethal in eukaryotes (31), and it has become clear that interaction of HBAs with their target is not the pharmacological equivalent of a knock-out. Instead, drug interaction with Hsp90 appears to alter some but not all of the cellular functions of this essential chaperone (32) and probably accounts for the observation that biological effects, such as destabilization of steroid receptors, can be achieved by drug exposures that are tolerable to cells in culture (Fig. 5) and whole animals (Fig. 6). Ongoing Phase I clinical trials have also found that micromolar peak concentrations of 17AAG can be achieved in human subjects without overt toxicity.⁴ Although quite selective in their interaction with Hsp90 class chaperones, the cellular effects of these agents are far from limited to destabilization of the ER and PR. They are known to disrupt the function of numerous Hsp90 client proteins, including receptor-linked kinases such as erbB-2 (33), and have shown *in vivo* activity in a colon cancer model (34). In an attempt to develop a GA derivative with greater selectivity for the ER, GA-estradiol conjugates have been reported that appear more active in stimulating the degradation of the ER than several other targets of GA *in vitro*, but no information on activity in animals has been reported (35). Although selectivity is often desirable, the fact that HBAs disrupt multiple cancer-associated targets may actually be of benefit therapeutically. Considerable cross-talk has been documented between the ER and PR pathways (36) and between the ER and erbB-2 pathways (37) in promoting breast cancer survival and growth. Simultaneous

⁴ C. Takimoto, University of Texas San Antonio, personal communication.

destabilization of these three receptors (and undoubtedly others) by HBAs may well provide more effective antitumor activity than targeting the ER alone.

Our *in vivo* data demonstrate that marked depletion of PR can be achieved after well-tolerated exposures to 17AAG (Fig. 6) and KF58333 (not shown). We adopted PR as a pharmacodynamic end point for these studies because PR expression is regulated by estrogen, and there is considerable evidence for cross-talk between the ER and PR in promoting breast cancer progression (38). At a technical level, PR was the preferred marker as well because ER levels are low in highly estrogen-supplemented mice, making their reliable detection difficult. Furthermore, serum albumins migrate very closely with the M_r 67,000 ER during SDS-PAGE electrophoresis and can interfere with its detection by immunoblotting. PR, on the other hand, is unregulated by estrogen supplementation, and its isoforms migrate well away from any interfering proteins. It is therefore a robust marker for drug effects on steroid receptor homeostasis in our animal model and in potential clinical trials in breast cancer patients in the future.

The activity demonstrated in Fig. 7 of 17AAG against established, hormone-responsive tumors is comparable with the activity reported for tamoxifen against MCF-7 tumors in another mouse model (39). The doses and schedules of 17AAG used for the experiments presented here were selected on the basis of preliminary work defining the maximum tolerated dose intensity in our SCID strain when bearing tumors. On the basis of our observations regarding the onset and duration of drug-induced effects *in vitro* (Fig. 5), a lower intensity dosing schedule spread over multiple weeks might be more appropriate if the agent is to be used more as a cytostatic modulator of hormone action than a direct cytotoxin. Although we have demonstrated that HBAs can effectively modulate steroid receptor levels, it is unlikely that the antitumor activity of HBAs resides solely in their destabilization of steroid receptors. Given the large number of intracellular Hsp90 clients altered by these drugs, other drug-sensitive substrates, such as erbB-2 or mutant p53 (40, 41), may well prove to be equally or more important to the overall anticancer activity of HBAs. Further work with hormone-independent breast tumors and recently described tamoxifen-resistant tumor models (42, 43) will be needed to define the best manner in which to exploit the novel biological activities of HBAs in the management of poor-prognosis breast cancer.

ACKNOWLEDGMENTS

We thank Marialouisa H. A. Lopez and Paul H. Cook for technical assistance. We thank Dan von Hoff for critical review prior to submission.

REFERENCES

- Osborne, C. K. Tamoxifen in the treatment of breast cancer. *N. Engl. J. Med.*, 339: 1609–1618, 1998.
- Katzenellenbogen, B. S. Antiestrogen resistance: mechanisms by which breast cancer cells undermine the effectiveness of endocrine therapy. *J. Natl. Cancer Inst.*, 83: 1434–1435, 1991.
- McGuire, W. L., Chamness, G. C., and Fuqua, S. A. Abnormal estrogen receptor in clinical breast cancer. *J. Steroid Biochem. Mol. Biol.*, 43: 243–247, 1992.
- Wijayaratne, A. L., Nagel, S. C., Paige, L. A., Christensen, D. J., Norris, J. D., Fowlkes, D. M., and McDonnell, D. P. Comparative analyses of mechanistic differences among antiestrogens. *Endocrinology*, 140: 5828–5840, 1999.
- Jordan, V. C. Tamoxifen: the herald of a new era of preventive therapeutics. *J. Natl. Cancer Inst.*, 89: 747–749, 1997.
- Smith, D. F., and Toft, D. O. Steroid receptors and their associated proteins. *Mol. Endocrinol.*, 7: 4–11, 1993.
- Smith, D. F., Whitesell, L., Nair, S. C., Chen, S., Prapapanich, V., and Rimerman, R. A. Progesterone receptor structure and function altered by geldanamycin, an hsp90-binding agent. *Mol. Cell. Biol.*, 15: 6804–6812, 1995.
- Stebbins, C. E., Russo, A. A., Schneider, C., Rosen, N., Hartl, F. U., and Pavletich, N. P. Crystal structure of an hsp90-geldanamycin complex: targeting of a protein chaperone by an anti-tumor agent. *Cell*, 89: 239–250, 1997.
- Roe, S. M., Prodromou, C., O'Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. Structural basis for inhibition of the Hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin. *J. Med. Chem.*, 42: 260–266, 1999.
- Grenert, J. P., Sullivan, W. P., Fadden, P., Haystead, T. A. J., Clark, J., Mimnaugh, E., Krutzsch, H., Ochel, H.-J., Schulte, T. W., Sausville, E., Neckers, L. M., and Toft, D. O. The amino-terminal domain of heat shock protein 90 (hsp90) that binds geldanamycin is an ATP/ADP switch domain that regulates hsp90 conformation. *J. Biol. Chem.*, 272: 23843–23850, 1997.
- Schulte, T. W., Akinaga, S., Soga, S., Sullivan, W., Stensgard, B., Toft, D., and Neckers, L. M. Antibiotic radicicol binds to the N-terminal domain of Hsp90 and shares important biologic activities with geldanamycin. *Cell Stress Chaperones*, 3: 100–108, 1998.
- Page, J., Heath, J., Fulton, R., Yalkowsky, E., Tabibi, E., Tomaszewski, J., Smith, A., and Rodman, L. Comparison of geldanamycin (NSC-122750) and 17-allylamino-geldanamycin (NSC-330507D) in rats. *Proc. Am. Assoc. Cancer Res.*, 38: 308, 1997.
- Shiotsu, Y., Neckers, L. M., Wortman, I., An, W. G., Schulte, T. W., Soga, S., Murakata, C., Tamaoki, T., and Akinaga, S. Novel oxime derivatives of radicicol induce erythroid differentiation associated with preferential G(1) phase accumulation against chronic myelogenous leukemia cells through destabilization of Bcr-Abl with Hsp90 complex. *Blood*, 96: 2284–2291, 2000.
- Whitesell, L., and Cook, P. Stable and specific binding of heat shock protein 90 by geldanamycin disrupts glucocorticoid receptor function in intact cells. *Mol. Endocrinol.*, 10: 705–712, 1996.
- Segnitz, B., and Gehring, U. The function of steroid hormone receptors is inhibited by the hsp90-specific compound geldanamycin. *J. Biol. Chem.*, 272: 18694–18701, 1997.
- Whitesell, L., Sutphin, P. D., Pulcini, E. J., Martinez, J. D., and Cook, P. H. The physical association of multiple molecular chaperone proteins with mutant p53 is altered by geldanamycin, an hsp90-binding agent. *Mol. Cell. Biol.*, 18: 1517–1524, 1998.
- Paine-Murrieta, G. D., Taylor, C. W., Curtis, R. A., Lopez, M. H. A., Dorr, R. T., Johnson, C. S., Funk, C. Y., Thompson, F., and Hersh, E. M. Human tumor models in the severe combined immune deficient (scid) mouse. *Cancer Chemother. Pharmacol.*, 40: 209–214, 1997.
- Bagatell, R., Paine-Murrieta, G., Taylor, C., Pulcini, E., Akinaga, S., Benjamin, I., and Whitesell, L. Induction of a heat shock factor 1-dependent stress response alters the cytotoxic activity of Hsp90 binding agents. *Clin. Cancer Res.*, 6: 3312–3318, 2000.
- Rose, C., and Mouridsen, H. T. Endocrine therapy of advanced breast cancer. *Acta Oncol.*, 27: 721–728, 1988.
- Nabholtz, J. M., Buzdar, A., Pollak, M., Harwin, W., Burton, G., Mangalik, A., Steinberg, M., Webster, A., and von Euler, M. Anastrozole is superior to tamoxifen as first-line therapy for advanced breast cancer in postmenopausal women: results of a North American multicenter randomized trial. *J. Clin. Oncol.*, 18: 3758–3767, 2000.
- Bonnetterre, J., Thurlimann, B., Robertson, J. F. R., Krzakowski, M., Mauriac, L., Koralewski, P., Vergote, I., Webster, A., Steinberg, M., and von Euler, M. Anastrozole *versus* tamoxifen as first-line

- therapy for advanced breast cancer in 668 postmenopausal women: results of the tamoxifen or arimidex randomized group efficacy and tolerability study. *J. Clin. Oncol.*, *18*: 3748–3757, 2000.
22. Picard, D., Khurshed, B., Garabedian, M. J., Fortin, M. G., Lindquist, S., and Yamamoto, K. R. Reduced levels of hsp90 compromise steroid receptor action *in vivo*. *Nature (Lond.)*, *348*: 166–168, 1990.
23. Binart, N., Lombes, M., and Baulieu, E-E. Distinct functions of the 90-kDa heat shock protein (hsp90) in oestrogen and mineralocorticosteroid receptor activity: effects of hsp90 deletion mutants. *J. Biochem.*, *311*: 797–804, 1995.
24. Fliss, A. E., Benzeno, S., Rao, J., and Caplan, A. J. Control of estrogen receptor ligand binding by Hsp90. *J. Steroid Biochem. Mol. Biol.*, *72*: 223–230, 2000.
25. Whitesell, L., Mimnaugh, E. G., De Costa, B., Myers, C. E., and Neckers, L. M. Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. *Proc. Natl. Acad. Sci. USA*, *91*: 8324–8328, 1994.
26. Pratt, W. B. The hsp90-based chaperone system: involvement in signal transduction from a variety of hormone and growth factor receptors. *Proc. Soc. Exp. Biol. Med.*, *217*: 420–431, 1998.
27. Caplan, A. J. Hsp90's secrets unfold: new insights from structural and functional studies. *Trends Cell Biol.*, *9*: 262–268, 1999.
28. Howell, A., Osborne, C. K., Morris, C., and Wakeling, A. E. ICI 182,780 (Faslodex): development of a novel "pure" antiestrogen. *Cancer (Phila.)*, *89*: 817–825, 2000.
29. Badia, E., Duchesne, M.-J., Sendlari, A., Fuentes, M., Giamarchi, C., Richard-Foy, H., Nicolas, J.-C., and Pons, M. Long-term hydroxytamoxifen treatment of an MCF-7-derived breast cancer cell line irreversibly inhibits the expression of estrogenic genes through chromatin remodeling. *Cancer Res.*, *60*: 4130–4138, 2000.
30. Jolly, C., and Morimoto, R. Role of the heat shock response and molecular chaperones in oncogenesis and cell death. *J. Natl. Cancer Inst.*, *92*: 1564–1572, 2000.
31. Borkovich, K. A., Farelly, F. W., Finkelstein, D. B., Taulien, J., and Lindquist, S. Hsp82 is an essential protein that is required in higher concentrations for growth of cells at higher concentrations. *Mol. Cell. Biol.*, *9*: 3919–3930, 1989.
32. Scheibel, T., Weikl, T., and Buchner, J. Two chaperone sites in Hsp90 differing in substrate specificity and ATP dependence. *Proc. Natl. Acad. Sci. USA*, *95*: 1495–1499, 1998.
33. Schnur, R. C., Corman, M. L., Gallaschun, R. J., Cooper, B. A., Dee, M. F., Doty, J. L., Muzzi, M. L., Moyer, J. D., DiOrto, C. I., Barbacci, E. G., *et al.* Inhibition of the oncogene product p185erbB-2 *in vitro* and *in vivo* by geldanamycin and dihydrogeldanamycin derivatives. *J. Med. Chem.*, *38*: 3806–3812, 1995.
34. Kelland, L. R., Sharp, S. Y., Rogers, P. M., Myers, T. G., and Workman, P. DT-diaphorase expression and tumor cell sensitivity to 17-allylamino, 17-demethoxygeldanamycin, an inhibitor of heat shock protein 90. *J. Natl. Cancer Inst.*, *91*: 1940–1949, 1999.
35. Kuduk, S. D., Zheng, F. F., Sepp-Lorenzino, L., Rosen, N., and Danishefsky, S. J. Synthesis and evaluation of geldanamycin-estradiol hybrids, bioorganic, and medicinal chemistry. *Letters*, *9*: 1233–1238, 1999.
36. Migliaccio, A., Piccolo, D., Castoria, G., Domenico, M. D., Bilancio, A., Lombardi, M., Gong, W., Beato, M., and Auricchio, F. Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. *EMBO J.*, *17*: 2008–2018, 1998.
37. Kurokawa, H., Lenferink, A. E., Simpson, J. F., Pisacane, P. I., Sliwkoski, M. X., Forbes, J. T., and Arteaga, C. L. Inhibition of HER2/neu (erbB-2) and mitogen-activated protein kinases enhances tamoxifen action against HER2-overexpressing, Tamoxifen-resistant breast cancer cells. *Cancer Res.*, *60*: 5887–5894, 2000.
38. Lange, C. A., Richer, J. K., and Horwitz, K. B. Hypothesis. Progesterone primes breast cancer cells for cross-talk with proliferative or antiproliferative signals. *Mol. Endocrinol.*, *13*: 829–836, 1999.
39. Osborne, C. K., Hobbs, K., and Clark, G. M. Effect of estrogens and antiestrogens on growth of human breast cancer cells in athymic nude mice. *Cancer Res.*, *45*: 584–590, 1985.
40. Blagosklonny, M. V., Toretzky, J., and Neckers, L. Geldanamycin selectively destabilizes and conformationally alters mutated p53. *Oncogene*, *11*: 933–939, 1995.
41. Whitesell, L., Sutphin, P., An, W. G., Schulte, T., Blagosklonny, M. V., and Neckers, L. Geldanamycin-stimulated destabilization of mutated p53 is mediated by the proteasome *in vivo*. *Oncogene*, *14*: 2809–2816, 1997.
42. Schafer, J. M., Lee, E. S., O'Regan, R. M., Yao, K., and Jordan, V. C. Rapid development of tamoxifen-stimulated mutant p53 breast tumors (T47D) in athymic mice. *Clin. Cancer Res.*, *6*: 4373–4380, 2000.
43. Naundorf, H., Becker, M., Lykkesfeldt, A. E., Elbe, B., Neuman, C., Buttner, B., and Fichtner, I. Development and characterization of a Tamoxifen-resistant breast carcinoma xenograft. *Br. J. Cancer.*, *82*: 1844–1850, 2000.

Clinical Cancer Research

Destabilization of Steroid Receptors by Heat Shock Protein 90-binding Drugs: A Ligand-independent Approach to Hormonal Therapy of Breast Cancer

Rochelle Bagatell, Omar Khan, Gillian Paine-Murrieta, et al.

Clin Cancer Res 2001;7:2076-2084.

Updated version Access the most recent version of this article at:
<http://clincancerres.aacrjournals.org/content/7/7/2076>

Cited articles This article cites 43 articles, 16 of which you can access for free at:
<http://clincancerres.aacrjournals.org/content/7/7/2076.full#ref-list-1>

Citing articles This article has been cited by 24 HighWire-hosted articles. Access the articles at:
<http://clincancerres.aacrjournals.org/content/7/7/2076.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://clincancerres.aacrjournals.org/content/7/7/2076>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.