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Inhibition of Heat Shock Protein 90 Function Down-Regulates Akt Kinase and Sensitizes Tumors to Taxol[1]

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ABSTRACT

The phosphatidylinositol 3′-kinase/Akt pathway is activated frequently in human cancer, and has been implicated in tumor proliferation, cell survival, and resistance to apoptotic stimuli. Akt forms a complex with heat shock protein (Hsp) 90 and Cdc37, and inhibitors of Hsp90 cause Akt degradation. 17-allylamino-17-demethoxygeldanamycin (17-AGG) is an Hsp90 inhibitor currently in Phase I clinical trial. 17-AAG inhibits Akt activation and expression in tumors, and has antitumor activity in breast cancer xenografts. The combination of 17-AAG and Taxol is synergistic, and 17-AAG sensitizes tumor cells to Taxol-induced apoptosis in a schedule-dependent manner. Transfection of membrane-bound p110 PI3k prevented 17-AAG inactivation of Akt and abrogated the enhancement of Taxol-induced apoptosis caused by the drug. 17-AAG and Taxol could be administered together at their maximally tolerated doses to tumor-bearing mice. Doses of 17-AAG that induce HER2 degradation and cause Akt inactivation but have no single agent activity were effective in sensitizing tumors to Taxol. Enhancement was schedule-dependent and maximal when Taxol and 17-AAG were administered on the same day. These results suggest that Hsp90 inhibitors can effectively suppress Akt activity in animal models of human cancer at nontoxic doses, thus sensitizing tumor cells to proapoptotic stimuli.

INTRODUCTION

Akt is a downstream target of PI3k,[3] and is responsible for many of the proliferative and antiapoptotic effects induced by binding of peptide growth factors to their transmembrane receptors (1). The pleckstrin homology domain in the Akt protein binds the products of PI3k activity, phosphatidyl inositol 3,4,5 phosphate, and is anchored to the plasma membrane where it is phosphorylated and activated by PDK1 and other protein kinases (2, 3). Induction of Akt activity by growth factors stimulates G_1 progression (4). In parallel to its stimulation of growth, Akt activation is responsible for desensitizing cells to the apoptotic stimuli induced by several growth factors, notably insulin-like growth factor I (5). This occurs via multiple mechanisms. Akt regulates several transcription factors that control the expression of genes involved in the process (forkhead, nuclear factor κB), and directly phosphorylates and inactivates components of the apoptotic machinery including Bax, Bad, and caspase 9 (6–8).

The ability of Akt to enhance proliferation and suppress apoptosis suggests that it might have strong oncogenic function. Indeed, Akt was identified initially as a retroviral-transforming gene and is activated by several mechanisms in human tumors (9). Akt is mutated and amplified in a variety of human tumors (10, 11). More often, activation of Akt in tumors results from upstream induction by overexpressed tyrosine kinases or mutations in genes that encode proteins that directly regulate its activity. The *PTEN* gene encodes a lipid phosphatase that dephosphorylates phosphatidyl inositol 3,4,5 phosphates and, thus, prevents Akt activation (12). *PTEN* is mutationally inactivated in several cancers, including glioblastoma and prostate cancer, and Akt is highly activated in tumors with defective *PTEN* function (13). The upstream activator of Akt, PI3k, is also altered in some human tumors (14). The frequency of Akt activation in human tumors and the functional consequences of its activation suggest that its pharmacologic inhibition may be a useful therapeutic strategy. With this goal in mind, direct inhibitors of PI3k activity are in development; selective inhibitors of Akt kinase have not yet been reported.

Akt was shown recently to associate with the chaperone proteins Hsp90 and Cdc37 in cells (15). In this complex, functional Hsp90 is required for Akt stability, and inhibitors of Hsp90 function cause Akt degradation (15). Hsp90 is also required for the stability or function of steroid receptors, the Raf and CDK4 serine kinases, and several transmembrane tyrosine kinases including HER2 (16–19). Hsp90 contains an ATP/ADP binding pocket in its NH_2-terminal domain that is required for its function (20). Several natural products, geldanamycin (GM), herbimycin A, and radicicol bind to this pocket and inhibit Hsp90 function (20–22). Treatment of cancer cells with GM causes the degradation of Raf, cdk4, and other targets that require Hsp90 for conformational maturation (18, 23). In breast cancer cells that overexpress HER2, Akt is activated and required for cellular proliferation (24). In these cells, inhibitors of Hsp90 cause the degradation of HER2 and the rapid inactivation of Akt (24). In addition, they also directly affect Akt stability and cause a loss of Akt expression (15).

In this report, we now demonstrate that, in breast cancer cells with high levels of HER2, inactivation of PI3k signaling is responsible for the enhancement of Taxol-induced apoptosis that results from Hsp90 inhibition. In mice, 17-AAG, a derivative of GM, currently in clinical trial, sensitizes xenograft tumors to Taxol in a schedule-dependent manner and without additive toxicity. Doses of 17-AAG sufficient to cause Akt inactivation in tumors but not tumor growth delay are capable of sensitizing tumors to the effects of Taxol. These data suggest that inhibitors of Hsp90 can effectively inactivate Akt in tumors, and their use in combination with proapoptotic therapies may represent a provocative new strategy for cancer treatment.

MATERIALS AND METHODS

Materials. 17-AAG (NSC 330507) and the EPL diluent were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, NCI. Drug was dissolved in DMSO to yield 50 mg/ml and 10 mM stock solutions, and stored at −20°C. The following antibodies were used for immunoblotting: Akt, P-Akt, P-4EBP1, P-GSK-3 (Cell Signaling, Beverly, MA), HER2, HER3, Raf-1 (Santa Cruz, Santa Cruz, CA), Hsp70, Hsp90 (StressGene, Victoria, British Columbia, Canada), and p85 subunit of PI3k (Upstate Biotechnology, Lake Placid, NY).

Cell Culture. The human breast cancer cell lines SKBr-3 and BT-474 (American Type Culture Collection, Manassas, VA) were maintained in a 1:1

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[3] The abbreviations used are: PI3k, phosphatidylinositol 3′-kinase; 17-AAG, 17-allylamino-17-demethoxygeldanamycin; GM, geldanamycin; Hsp, heat shock protein; PDK1, phosphatidyl inositol, 3′ kinase 1; cdk, cyclin-dependent kinase; NCI, National Cancer Institute; EPL, egg-phospholipid; MTD, maximally tolerated dose.

mixture of Dulbecco's modified Eagle's medium:F12 supplemented with 2 mM glutamine, 50 units/ml penicillin, 50 units/ml streptomycin, and 10% heat-inactivated fetal bovine serum (Gemini Bioproducts, Calabasa, CA) and incubated at 37°C in 5% CO_2. The membrane-bound PI3k construct was provided by Julian Downward (Cancer Research UK, London, United Kingdom). The COOH-terminal farnesylation signal from H-Ras was fused to p110 (myc-tagged p110-CAAX in pSG5 vector; Ref. 25). Two million cells were transfected with 2 μg of p110-CAAX DNA or vector control using 10 μl Lipofectin reagent (Life Technologies, Inc., Rockville, MD). Experiments were performed 36 h after transfection. For apoptotic scoring, cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Nuclei were stained with 0.5 μg/ml bis-benzimide (Hoechst 33258), and cell were analyzed by confocal and epifluorescent microscopy. Cells were scored for mitosis, apoptosis, or interphase, and indices were quantified by counting 300–500 cells manually in three different fields and reported as the percentage of total cells. Treatment groups were compared using the stratified rank test, where the strata corresponded to three repetitions of the experiment.

Akt kinase activity was assayed using the Akt Kinase kit (Cell Signaling). Akt was immunoprecipitated from 200 μg of total protein lysates with immobilized Akt beads, washed twice with lysis buffer, then twice with kinase buffer [25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$]. Complexes were resuspended in 40 μl of kinase buffer and 200 μM of ATP, and 1 μg of GSK-3 substrate was added. Assays were performed at 30°C for 30 min and stopped with sample buffer. Reaction mixture was denatured by boiling, resolved on a 10% SDS-PAGE, and transferred to nitrocellulose. Western blots were performed with P-GSK-3 antibody (Cell Signaling).

Animal Studies. Four to 6-week old *nu/nu* athymic female mice were obtained from the NCI-Frederick Cancer Center and maintained in ventilated caging. Experiments were carried out under an Institutional Animal Care and Use Committee approved protocol, and institutional guidelines for the proper and humane use of animals in research were followed. One $\times 10^7$ BT-474 tumor cells were injected together with reconstituted basement membrane (Matrigel; Collaborative Research, Bedford, MA). Before tumor cell inoculation, 0.72 mg/day 17β-estradiol pellets (Innovative Research of America, Sarasota, FL) were placed s.c. Mice with established tumors 5–6 mm in diameter were selected for study ($n = 10$–15/treatment group). All of the mice received Augmentin (Amoxicillin/Clavulanate potassium; SmithKline Beecham) in their drinking water while on therapy. Tumor dimensions were measured with vernier calipers, and tumor volumes were calculated with the formula: $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2. Mice were followed until tumor volumes reached Institutional Animal Care and Use Committee guidelines and were monitored for toxicity at least twice each week. Treatment groups were compared using the Wilcoxon rank sum test as described previously (26). Mice were sacrificed by CO_2 euthanasia.

Before administration, 17-AAG was dissolved in an EPL vehicle developed for this purpose by the NCI. Taxol (paclitaxel; Bristol-Myers Squibb, Princeton, NJ) was diluted at minimum 1:3 in normal saline. Mice were treated with Taxol at a dose of 25 mg/kg every 5–7 days for five cycles, 17-AAG at doses of 10–150 mg/kg for 1–3 days each week, or the vehicles only as control. In experiments designed to define the pharmacodynamic effects of 17-AAG on Hsp90 client proteins in tumor tissue, mice with established tumors were treated with 17-AAG at the doses specified or with EPL vehicle alone. To prepare lysates from xenograft tumors, tumor tissue was homogenized in 2% SDS lysis buffer [50 mM Tris-HCl (pH 7.4) and 2% SDS] and then processed as described previously (26).

RESULTS

Inhibition of Akt by 17-AAG Sensitizes Tumor Cells to Taxol. In breast tumors with high levels of HER2 expression, Akt is activated by a HER2/HER3, PI3k-dependent pathway (24). Treatment of BT-474 and SKBr-3 cells with 17-AAG causes degradation of HER2 and a rapid (within 1 h) inactivation of Akt. Even so, in these cells, 17-AAG induces only a modest degree of apoptosis 48–72 h after drug addition. 17-AAG does sensitize these cells to induction of apoptosis by taxanes (27). To determine whether sensitization is because of PI3k/Akt inhibition, a constitutively active, membrane-bound form of p110 PI3k was transfected into SKBr-3 cells. Transfection of p110-CAAX caused an increase in basal Akt activity and attenuated the effects of 17-AAG on Akt kinase activity for up to 24 h (Fig. 1*A*). Because Akt is an Hsp90-dependent protein, a gradual loss of Akt kinase activity paralleling the loss of Akt protein expression is observed in p110-transfected cells (Fig. 1*A*).

We found that p110-CAAX had minimal effect on induction of apoptosis by Taxol but prevented its enhancement by 17-AAG (Fig. 1*B*). At 18 h, 8% of mock-transfected cells treated with 100 nM 17-AAG and 18% of the cells treated with 10 nM Taxol were apoptotic as determined by bis-benzimide staining. After treatment with the combination, 28% of mock-transfected cells were apoptotic. Transfection of p110-CAAX reduced the percentage of apoptotic cells treated with the combination to 17% without significantly altering the percentage of apoptotic cells in the Taxol and 17-AAG groups. Thus, 17-AAG sensitizes tumor cells to taxanes by inactivating PI3k-dependent pathways.

17-AAG Causes HER2 Degradation and Akt Inactivation in Human Breast Cancer Xenografts. In murine systems, 17-AAG induces HER2 degradation and Akt inactivation in breast cancer xenografts at nontoxic doses (24, 26). The effects on target protein

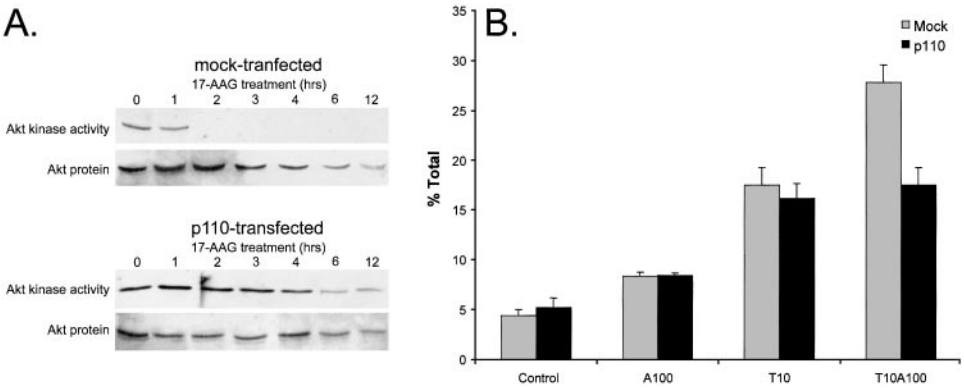

Fig. 1. Inhibition of Akt by 17-AAG sensitizes tumor cells to Taxol. *A*, Akt kinase assay of mock- and p110-CAAX-transfected SKBr-3 cells treated with 17-AAG. Transfection of p110-CAAX causes an increase in basal Akt activity and prevents the rapid loss of Akt activity that results from 17-AAG treatment. Because Akt is an Hsp90-dependent protein, a gradual loss of Akt kinase activity paralleling the loss of Akt protein expression is observed in p110-transfected cells. Akt activity in the p110-CAAX-transfected cells treated with 17-AAG remains above the Akt basal rate of mock-transfected cells for up to 24 h. *B*, SKBr-3 cells were treated with DMSO, 17-AAG 100 nM, Taxol 10 nM, or the combination. Transfection of p110-CAAX had minimal effect on the induction of apoptosis by Taxol ($P = 0.14$) but significantly attenuated the sensitization observed with 17-AAG ($P < 0.001$). Treatment with the combination of Taxol and 17-AAG resulted in a significant increase in apoptosis *versus* Taxol alone in mock-transfected (27.7 $\pm$ 1.8% for the combination *versus* 17.7 $\pm$ 1.7% for Taxol alone; $P < 0.001$) but not in p110-transfected cells (17.5 $\pm$ 1.7 for the combination *versus* 16.1 $\pm$ 1.5 for Taxol alone; $P = 0.168$). *Bars*, $\pm$SE.

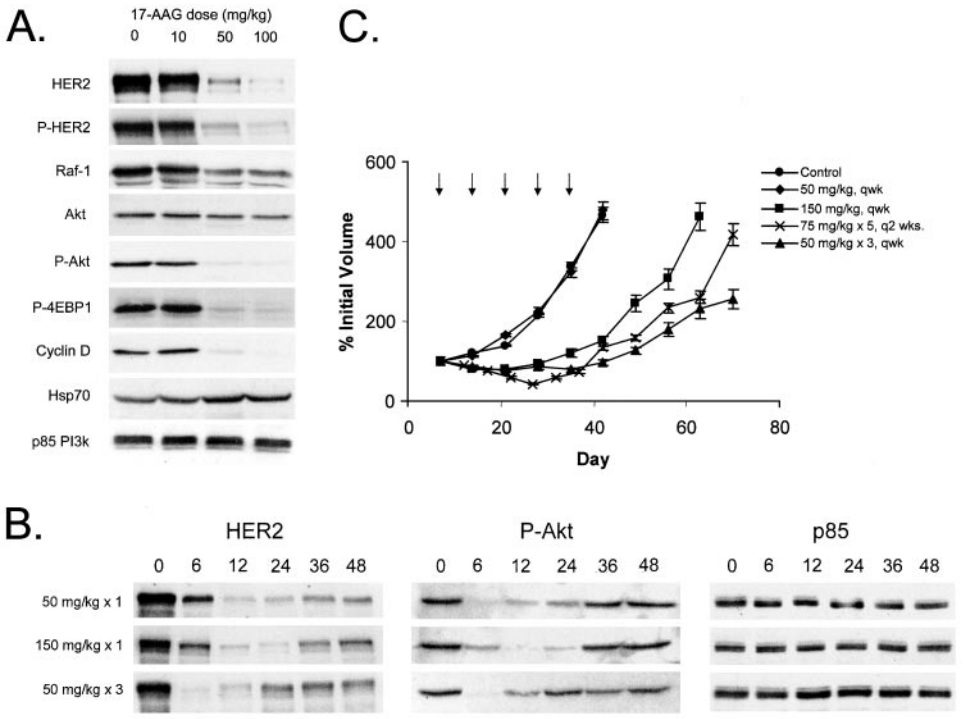

Fig. 2. 17-AAG induces HER2 degradation and Akt inactivation, and inhibits the growth of breast cancer xenografts at nontoxic doses. *A*, 17-AAG degradation of HER2 and inactivation of Akt are dose-dependent. Immunoblot of BT-474 tumors from mice treated with EPL vehicle alone (0), and 17-AAG 10, 50, and 100 mg/kg. *B*, the kinetics of HER2 degradation and Akt inactivation in BT-474 xenografts from mice treated with 17-AAG. Treatment with 17-AAG at doses >50 mg/kg did not prolong the duration of HER2 loss or Akt inactivation. Times indicate hours post-treatment. *C*, the effects of 17-AAG on BT-474 tumor growth are dose- and schedule-dependent. Treatment with 17-AAG 50 mg/kg once weekly had no effect on tumor growth *versus* vehicle treated controls ($P = 0.797$). Three and 5 consecutive day treatment cycles were most effective ($P < 0.001$ for both the 3 and 5 consecutive day treatment group *versus* vehicle-treated controls). Treatment with 150 mg/kg had intermediate activity ($P = 0.049$). *Arrows* identify weeks of treatment. *Bars*, ±SE.

expression are dose- and schedule-dependent (Fig. 2*A*). A single 50 mg/kg dose of 17-AAG causes a 91% decline in HER2 expression and a >95% reduction in Akt activation. The maximum effect occurred 12 h after treatment and was followed by a gradual rise in HER2 expression toward baseline levels (Fig. 2*B*). Treatment of mice with a higher dose of 17-AAG (150 mg/kg) resulted in only a modest increase in the magnitude of HER2 degradation (99% at 12 h) and did not prolong the duration of HER2 loss. All of the doses >50 mg/kg were sufficient to inhibit Akt phosphorylation by >95% (Fig. 2*A*). A more prominent dose response was noted with other Hsp90 client proteins such as Raf and Akt especially at later time points (Fig. 2*A*; data not shown). Additional doses of 17-AAG failed to prolong the duration of HER2 loss and Akt inactivation after the final dose of therapy (Fig. 2*B*). A trend toward an earlier rise in HER2 expression after repeated administrations was noted.

17-AAG Inhibits the Growth of Xenograft Tumors in a Dose- and Schedule-dependent Manner. We compared the effects of 17-AAG on target proteins with its antitumor effects. The MTD of 17-AAG in tumor-bearing mice varies from 75 to 150 mg/kg and is dependent on the schedule of its administration (26). A correlation between dosing frequency and toxicity has also been noted in the ongoing Phase I trials of this agent. Intermittent (weekly) dosing is associated with less toxicity (MSKCC trial 99-037) than are schedules in which the drug is given for 3 or 5 consecutive days. We found that treatment with 50 mg/kg 17-AAG three times per week for 5 weeks or 75 mg/kg on days 1–5 and a second cycle on days 15–19 resulted in a significant delay in xenograft tumor growth (Fig. 2*C*). Whereas a single 50 mg/kg dose of 17-AAG induced the degradation of Hsp90 client proteins such as HER2, weekly treatment with 17-AAG at a dose of 50 mg/kg did not inhibit tumor growth (Fig. 2). Weekly treatment of mice at the MTD (150 mg/kg/wk) did result in modest tumor growth delay but was less effective than lower doses administered for 3 consecutive days each week or 5 consecutive days every other week. Thus, inhibition of target protein expression is not sufficient to inhibit tumor growth. Furthermore, whereas weekly dosing is associated with less toxicity, greater antitumor activity was observed with more frequent dosing schedules.

17-AAG Sensitizes Tumor Xenografts to Taxol. As inhibition of the PI3k/Akt pathway by 17-AAG sensitizes tumor cells in culture to Taxol, we sought to determine whether this combination could be administered *in vivo* to similar effect. We treated mice with established BT-474 tumors with single weekly doses of 25 mg/kg Taxol and 50 mg/kg 17-AAG. A weekly dose of 50 mg/kg 17-AAG was sufficient to cause a >90% reduction in HER2 expression and Akt activation but did not inhibit tumor growth (Fig. 2). We found that 50 mg/kg 17-AAG weekly significantly enhanced the antitumor effects of Taxol (Fig. 3; Table 1). Tumors from mice treated with the combination demonstrated a mean 83% reduction in volume on day 35 (1 week after the fifth and final dose of therapy). Twenty percent of mice in the combination group demonstrated a complete response to therapy. In contrast, mice treated with single-agent Taxol (25 mg/kg/wk) demonstrated tumor stability during therapy. As stated above, 17-AAG alone at this dose and schedule had no antitumor activity.

In this experiment, the combination could easily be administered without evidence of additive toxicity. In initial experiments designed to determine the tolerability of the combination, two early deaths were noted when 17-AAG and Taxol were administered concurrently on the same day. This toxicity was suspected to be secondary to the simultaneous administration of the Cremophor EL (polyoxyethylated castor oil) and DMSO vehicles by i.p. injection. In subsequent experiments, a 30-min delay separated the 17-AAG and Taxol treatments. With this delay, no toxicity was noted in mice treated with the combination at the single agent MTD of each drug.

To determine whether higher doses of 17-AAG were more effective in sensitizing tumors to Taxol, mice were treated with Taxol and 150 mg/kg/wk of 17-AAG as a single bolus (150 × 1) or in three divided doses of 50 mg/kg/day (50 × 3). Regressions of 87% and 86% by volume were seen in the 150 × 1 and 50 × 3 combination arms, respectively (Fig. 3; Table 1). These results were not statistically different from the 83% regression observed with the 50 × 1 schedule. A higher complete response rate compared with the weekly 50 mg/kg dose was observed (40% for both 150 × 1 and 50 × 3 *versus* 20% for 50 × 1). These data suggest that whereas a dose of 17-AAG sufficient

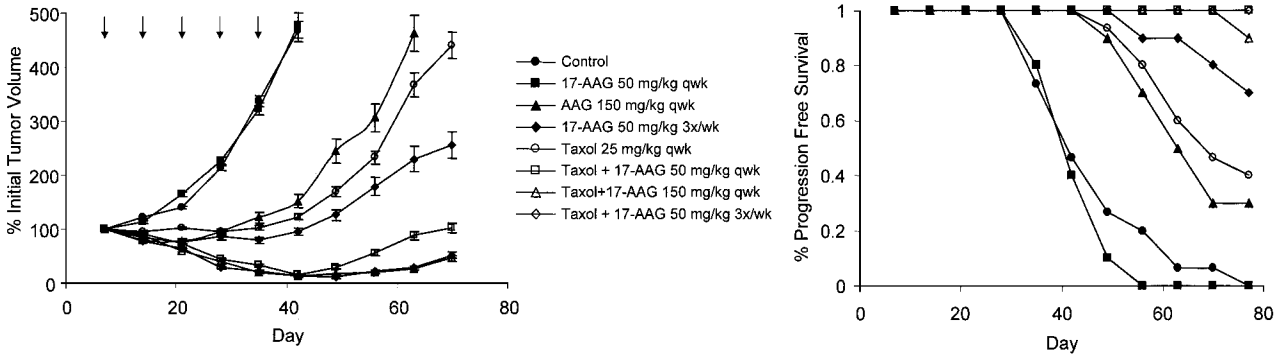

Fig. 3. 17-AAG sensitizes BT-474 xenograft tumors to Taxol. Fifty mg/kg of 17-AAG, a dose sufficient to cause HER2 degradation and Akt inactivation, had no activity as a single agent but did sensitize tumors to Taxol. *Arrows* represent weeks of treatment. *Bars*, ±SE.

Table 1 *17-AAG enhanced the activity of Taxol*

Mean change in initial tumor volume at day 35 (1 week after completing treatment) and the percentage of complete responses in each treatment arm.

	Mean % volume change (day 35)	% CR[a]	P *vs.* Taxol	P *vs.* 17-AAG 50 mg/kg 3x/wk
Vehicle controls	466	-		
17-AAG 50 mg/kg qwk	448	-		
17-AAG 150 mg/kg qwk	152	10%		
17-AAG 50 3×/wk	−5	20%		
Taxol	23	6.7%		
Taxol + 17-AAG 50 mg/kg qwk	−83	20%	0.02	< 0.01
Taxol + 17-AAG 150 mg/kg qwk	−87	40%	< 0.01	< 0.01
Taxol + 17-AAG 50 mg/kg 3x/wk	−86	40%	< 0.01	< 0.01

%CR, % complete responses. $n = 15$ for the vehicle and Taxol-treated groups, $n = 10$ for the 17-AAG and combination groups.

to cause maximal Akt inactivation can enhance the effects of Taxol, higher doses of 17-AAG do result in a small incremental increase in efficacy.

Enhancement of Taxol Activity by 17-AAG Was Schedule-dependent. We have demonstrated previously in tissue culture systems that enhancement of Taxol-induced apoptosis by 17-AAG is schedule-dependent (27). Treatment of BT-474 cells with 17-AAG causes a G_1 growth arrest, whereas Taxol arrests these cells in mitosis (28). In cells with wild-type retinoblastoma protein expression such as BT-474, maximal enhancement in tissue culture systems occurs when Taxol treatment is concurrent with or precedes treatment with 17-AAG (27). Pretreatment of these cells with 17-AAG before Taxol results in G_1 arrest and reduced Taxol-induced cytotoxicity and apoptosis.

To assess the effect of schedule on the antitumor activity of the combination *in vivo*, mice were treated with 17-AAG administered sequentially on the same day as Taxol, or 24 h before or subsequent to Taxol. The combinations were administered every 5 days for five cycles. All three of the combination arms were well tolerated, and superior to Taxol and 17-AAG as single agents (Fig. 4). The most effective combination schedule was the sequential administration of the two agents on the same day. With this schedule, maximal mean tumor regression of 88% was noted on day 37 compared with 17% with Taxol alone. Treatment with 17-AAG either 24 h before or 24 h after Taxol was less effective than administering the agents concurrently. Mice treated with 17-AAG and Taxol on the same day had a greater maximal mean tumor regression (88%) and a more durable treatment response than mice treated with 17-AAG 24 h before (63%) or 24 h after (72%) Taxol. These data strongly suggest that a mechanistic interaction between the drugs is responsible for sensitization.

DISCUSSION

Hsp90 is a chaperone required for the refolding of proteins in cells exposed to environmental stress and for the conformational maturation of several key regulatory proteins, including steroid receptors, HER2, and the Raf, PDK1, Akt and cdk4 kinases (15, 26, 29–31). Several natural products, including GM and radicicol, bind specifically to the ATP-binding site in the NH_2-terminal portion of Hsp90, and inhibit its function (20–22). Inhibition of Hsp90 function leads to the degradation of its client proteins, and the retinoblastoma-dependent G_1 arrest and subsequent differentiation of several classes of epithelial tumor cells (28, 32). G_1 arrest results from degradation of cdk4 kinase and from inhibition of an Akt dependent-signaling pathway that is required for expression of D-cyclins (33). The latter effect is especially prominent in breast cancer cells that overexpress HER2 (24).

In this paper, we show that 17-AAG, the Hsp90 inhibitor in clinical trial, causes the degradation of HER2, Akt, and Raf in human HER2-dependent breast cancers grown as murine xenografts. Degradation of these proteins is dose-dependent. Inhibition of HER2 leads to a rapid and marked loss in Akt activity in the tumor, as measured by loss of serine-473 phosphorylation and inhibition of the phosphorylation of Akt substrates such as 4-EBP1. Inhibition of Akt occurs at nontoxic doses of 17-AAG within 1 h of drug administration and persists up to

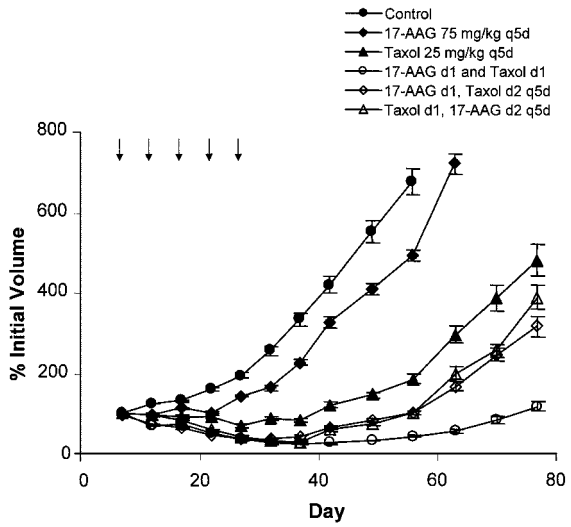

Fig. 4. Enhancement of Taxol by 17-AAG was schedule-dependent. Treatment with 17-AAG 24 h before or 24 h after Taxol was less effective than administration of both compounds in sequence on the same day. *Arrows* represent cycles of treatment. *Bars*, ±SE.

24 h. Thus, Akt kinase can be inhibited in tumors with safe doses of an Hsp90 inhibitor, at least in breast cancers that overexpress HER2. Akt inhibition correlated with antitumor activity and sensitization of the tumor to the cytotoxic effects of Taxol.

Inhibition of Akt activity is likely to be because of two causes. Both Akt and PDK1 have been shown recently to associate with Hsp90 in a complex that is required for their stability, insofar as Hsp90 inhibitors cause their ubiquitination, targeting to the proteasome and degradation (15, 31). However, the degree of Akt kinase inhibition demonstrated in HER2-dependent tumors is out of proportion to the loss in Akt expression. This is because of the degradation of HER2 protein in response to 17-AAG. We have shown in tissue culture models that Akt kinase is activated in breast cancer cell that overexpress HER2 and that in these cells 17-AAG causes rapid inhibition of the Akt kinase before any effects on Akt protein expression (24). This loss of kinase activity results from degradation of HER2, loss of HER3 phosphorylation, and an attendant decline in the docking of active PI3k to HER3.

Activation of Akt in tumors leads to deregulation of growth and to desensitization of the tumor cell to proapoptotic stimuli (34). 17-AAG causes the dose- and schedule-dependent inhibition of BT-474 tumor growth, with higher doses and more frequent scheduling causing tumor regression and a few complete responses. Doses required for antitumor activity correlate with those required to induce a stress response (increases in Hsp70 expression) and degradation of the Hsp90 target proteins, including HER2, Akt, and Raf. The role contributed by inhibition of each of these targets is not clear, although it is likely that HER2 inhibition is important. The steady state level of each of the target proteins begins to rise 24 h after administration of the drug, although levels of HER2 expression have still not returned to baseline even 48 h after drug addition. Thus, antitumor effects can be accomplished with episodic inhibition of the drug targets. Furthermore, qualitative inhibition of client protein expression is not sufficient for antitumor activity. This has implications for current pharmacodynamic studies of targeted drugs, which assay loss of expression of immunohistochemical markers of target protein activity.

Akt kinase inhibits induction of apoptosis by a variety of stimuli. We have shown previously in tissue culture models that 17-AAG sensitizes cells to induction of apoptosis by taxanes. We now show that 17-AAG and Taxol have synergistic activity in the BT-474 breast cancer xenograft model. The synergy is schedule-dependent and occurs when the drugs are given on the same day; administration of 17-AAG 24 h before or after the Taxol has much less activity. These data suggest that the effect of the combined drugs is secondary to a mechanistic interaction. Indeed, we have shown that, in tissue culture, introduction of a constitutively active form of PI3k has minimal effect on Taxol-induced apoptosis but prevents sensitization by 17-AAG.

Thus, it is likely that inhibition of Akt kinase by 17-AAG is responsible, at least in part, for its sensitization of cells to taxanes. It is difficult to extrapolate results obtained in tissue culture to the xenograft system. The doses and schedules of 17-AAG required for Akt inhibition also result in sensitization. Furthermore, administration of single doses of 17-AAG that, by themselves, have no antitumor activity but do result in Akt kinase inhibition for 24 h, have significant synergistic activity in combination with taxanes. These findings have important implications for the development of strategies for using signal transduction inhibitors in combination with cytotoxics. Current paradigms are based on the idea that signaling inhibitors are cytostatic and that continuous inhibition of the target is required. This idea is not consistent with our data. The pattern of target inhibition that will cause optimal antiproliferative effects may be different from that required to sensitize cells to proapoptotic agents, or to inhibit angiogenesis or other stromal interactions. Furthermore, continuous administration of a cytostatic agent that causes G_1 cell cycle arrest may attenuate or prevent the antitumor effects of antimitotics and other cell cycle-specific agents. Lastly, as Hsp90 inhibitors induce Hsp90 and Hsp70 expression, continuous administration may accelerate the development of resistance. In fact, we observed that the kinetics of HER2 rise was accelerated after 3 days of drug administration.

Hsp90 has multiple intracellular client proteins that play important roles in mediating mitogenic signaling and cell survival under stressful conditions. This suggests that Hsp90 inhibition may be associated with severe toxicity. Surprisingly, this is not the case. Doses of 17-AAG that induce a stress response, inhibit client protein expression, and cause antitumor activity have little or no toxicity. Furthermore, there is no additive toxicity with Taxol, and this combination can be given easily to mice. The data suggest that Akt inhibition in tumors can be achieved clinically and that 17-AAG may be useful therapeutically in tumors in which activation of this pathway is required for proliferation or suppression of apoptosis. The schedule and dose dependence of its sensitization of tumors to Taxol provides useful lessons for the development of signaling inhibitors in combination with cytotoxic therapy.

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