Advances in Brief

Modulation of Hsp90 Function by Ansamycins Sensitizes Breast Cancer Cells to Chemotherapy-induced Apoptosis in an RB- and Schedule-dependent Manner[1]

See *The Biology Behind:* E. A. Sausville, Combining Cytotoxics and 17-Allylamino, 17-Demethoxygeldanamycin: Sequence and Tumor Biology Matters. Clin. Cancer Res., *7:* 2155–2158, 2001.

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Abstract

17-allyl-aminogeldanamycin (17-AAG) is an ansamycin antibiotic that binds to a highly conserved pocket in the Hsp90 chaperone protein and inhibits its function. Hsp90 is required for the refolding of proteins during cellular stress and the conformational maturation of certain signaling proteins. 17-AAG has antitumor activity in cell culture and animal xenograft models and is currently in clinical trial. It causes an RB-dependent G_1 arrest, differentiation, and apoptosis. RB-negative cells arrest in mitosis and undergo apoptosis. Hsp90 plays an important role in the cellular response to environmental stress. Therefore, we tested whether the regulation of Hsp90 function by 17-AAG could sensitize cells to cytotoxic agents. 17-AAG sensitized tumor cells to Taxol and doxorubicin. Taxanes cause growth arrest in mitosis and apoptosis. The addition of 17-AAG to cells after exposure to Taxol significantly increased both the activation of caspases 9 and 3 and apoptosis. In cells with intact RB, exposure to 17-AAG before Taxol resulted in G_1 arrest and abrogated apoptosis. Schedule dependence was not seen in cells with mutated RB, because both agents blocked cells in mitosis. Schedule- or RB-dependence was also not observed when cells were treated with 17-AAG and doxorubicin, a DNA-intercalating agent that acts on different phases of the cell cycle. These findings suggest that inhibition of Hsp90 function by 17-AAG enhances the apoptotic effects of cytotoxic agents. The sequence of drug administration and the RB status significantly influence efficacy.

Introduction

The ansamycin antibiotic geldanamycin (GA) and its modified derivative 17-AAG[3] bind to a conserved pocket in the Hsp90 protein and inhibit its function (1, 2). Hsp90 is important for the maturation of a subset of cellular proteins that are dependent on this chaperone and the refolding of proteins in cells exposed to stress (3, 4). Among the most sensitive targets of the drugs are the HER kinases, Raf, met tyrosine kinase, and steroid receptors (5–8). Treatment of cancer cells with GA and 17-AAG causes an RB-dependent G_1 block that is mediated by selective down-regulation of pathways responsible for induction of cyclin D-cyclin-dependent kinase (cdk) 4,6 protein kinase activity (9). Cell cycle arrest is followed by differentiation and apoptosis (10). In cells with mutated RB, G_1 progression is unaffected by ansamycins. Instead, these cells undergo cell cycle arrest in mitosis followed by apoptosis (10). 17-AAG has antitumor activity in a variety of breast cancer cells lines and animal xenograft models and is now in clinical Phase I trials (11). Its mechanism of action implies that it might be effective in tumors dependent on HER2, AR, or *met* or in RB-negative tumors. In fact, preliminary data suggest that breast tumors that overexpress HER2 are especially sensitive to 17-AAG.

The regulation of Hsp90 function by 17-AAG suggests another potential strategy for its clinical use. Hsp90 plays an important role in the cellular response to environmental stress induced by heat, radiation, or toxins (12, 13). The ability to induce Hsp90 may promote survival in cells exposed to stress (14, 15). Therefore, the regulation of Hsp90 function by 17-AAG might render cancer cells more susceptible to cytotoxic agents. We tested this hypothesis by treating breast cancer cells with 17-AAG in combination with the cytotoxic agents Taxol and doxorubicin. Both of these agents induce apoptosis in breast cancer cells. Our results showed that the addition of 17-AAG sensitized cancer cells to apoptosis induced by Taxol. Taxol-induced cell cycle arrest occurred in mitosis and was followed by apoptosis. 17-AAG sensitized cells to Taxol-induced apoptosis when the drugs were given together or when 17-AAG treatment followed exposure to Taxol. The effects of the drug

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[3] The abbreviations used are: 17-AAG, 17-allyl-aminogeldanamycin; RT, room temperature; NMP, nuclear matrix protein; ER, estrogen receptor; RB, retinoblastoma protein; cdk; cyclin-dependent kinase.

combination were synergistic. In contrast, pretreatment of breast cancer cells with 17-AAG resulted in G_1 arrest and abrogated the apoptotic effects of Taxol. This schedule dependence was not observed in RB-negative cells in which both agents caused mitotic arrest. Synergistic effects were also found when 17-AAG was added to doxorubicin, an agent that affects all of the phases of the cell cycle. These effects were independent of schedule and RB status. Therefore, 17-AAG may significantly enhance the antitumor activity of Taxol and doxorubicin in a schedule- and RB-dependent manner.

Materials and Methods

Cell Culture and Growth Assays. SKBr-3, BT-474, BT-549, MDA-361, MDA-453, T 47-D, MCF-7, and MDA-MB-468 cells were obtained from the American Type Culture Collection. Cells were maintained in a 1:1 mixture of DMEM: Ham's F-12 media supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 4 mM glutamine, and 10% heat-inactivated fetal bovine serum and incubated at 37°C in 5% CO_2. Growth assays were performed by seeding 2000 cells/well in 96-well, flat-bottomed plates and treating them with drug 24 h before drug treatment. Cells were incubated with Taxol or doxorubicin for 4 h. After the removal of the cytotoxic agent, cells were incubated with vehicle or 17-AAG (16 wells/concentration) in complete medium (200 μl/well) for 96 h and assayed by the Alamar Blue viability test as described elsewhere (16). Isobologram analysis was performed using the CalcuSyn software program designed by Ting-Chao Chou in collaboration with BIOSOFT.

Reagents. Doxorubicin (Pharmacia), cisplatin (Bristol-Laboratory), and Taxol (Mead-Johnson) were diluted in media to achieve the desired concentration. 17-AAG (provided by Edward Sausville, National Cancer Institute, Bethesda, Maryland) was dissolved in DMSO at a 10 mM stock solution. Final working solutions were diluted in media to contain less than 0.01% of DMSO. Bis-benzimide (Hoechst 33258) and β-tubulin monoclonal antibody were purchased from Sigma Chemical Co. Chemicals and Alamar Blue were purchased from Accumed. Reagents for caspase activation assays and for the nuclear matrix protein ELISA were purchased from Oncogene Research Laboratory. Polyclonal antibodies were from Upstate Biotechnology, Inc. (p85) and from Santa Cruz Biotechnology (HER2 and Raf).

Immunoblotting. Cells were harvested, washed twice in PBS, and lysed in NP40 lysis buffer [50 mM Tris-Cl (pH 7.4), 1% NP40, 40 mM NaF, 150 mM NaCl, 10 mM/ml of each NA_3VO_4, phenylmethylsulfonyl fluoride, and DTT, and 1 μg/ml each of leupeptin, aprotinin, and soybean trypsin inhibitor)]. Lysate (50 μg) was loaded onto 7–10% SDS-PAGE minigels. As described previously (10), proteins were transferred to nitrocellulose membranes and incubated with primary and secondary antibodies. Proteins were visualized by chemiluminescence (enhanced chemiluminescence; Amersham Corp.) on Bio-Max film (Eastman Kodak).

Cell Cycle Analysis. Cell nuclei were prepared by the method described by Nusse *et al.* (17), and cell cycle distribution was determined by flow cytometric analysis using ethidium bromide as a measure of DNA content. Cell sorting was performed using a Becton Dickinson fluorescence-activated cell sorter cytometer (San Diego, CA).

Drug Treatment for Apoptosis Induction. Cells were seeded in 10-cm cell culture plates at a density of 10^6 cells/dish 24 h before drug exposure. Cells were treated with Taxol or doxorubicin for 4 h, and then drug was removed. 17-AAG was added either 24 h or 12 h prior, at the same time or immediately after the cytotoxic agents. Apoptosis was assessed at the indicated times after removal of the cytotoxic agent.

Assessment of Apoptosis and Mitosis. After drug treatment, adherent and supernatant cells were harvested and fixed with 3.5% paraformaldehyde for 10 min at RT and permeabilized with 0.2% Triton X-100 for 10 min at RT in 15-ml Falcon tubes. For tubulin staining, cells were incubated with a monoclonal β-tubulin antibody for 1 h at RT and then labeled with an Alexa-488-conjugated secondary antibody for 1 h at RT. Nuclei were stained with 0.5 μg/ml bis-benzimide (Hoechst 33258). Cell suspensions were then placed on glass slides and analyzed by confocal or conventional epifluorescent microscopy. Cells were scored for mitosis, apoptosis, or interphase by the following criteria. Cells in interphase had a fine distribution of tubulin in the cytoplasm and a mildly convoluted nucleus with fine chromatin structures. Untreated and 17-AAG-treated mitotic cells showed DNA condensation with tubulin structures emanating from two poles on opposing sites. Mitotic figures in Taxol-treated cells were characterized by chromatin condensation with disordered mitotic spindles or multiple asters (18). Cells with invagination, budding of the nucleus, breakdown of the DNA structure, or DNA fragmentation were classified as apoptotic (19). Indices were quantified by counting 200 cells manually in five different fields and reported as percentage of total cells. Each experiment was repeated at least three times. Statistical analysis was performed using Student's paired *t* test.

Caspase activity for caspases 9 and 3 was measured in lysates of treated cells measuring the increase of a fluorescent-labeled substrate of the enzymes. Each caspase is specific for its preference for a four-amino acid recognition motif after aspartate in their substrate (LEHD for caspase 9; DEVD for caspase 3). The substrate was labeled with the fluorescent molecule 7-amino-4-trifluoromethylcoumarin, and the reaction was measured by a blue to green shift in the fluorescence upon cleavage of the 7-amino-4-trifluoromethylcoumarin fluorophore. These tests were standardized with the respective recombinant caspases and caspase inhibitors. Cell death was also quantified by the release of NMPs. These proteins are highly insoluble but become soluble upon cell death and can be detected in the cell culture supernatant. NMP ELISA was performed using an assay from Oncogene Research Lab (QIA #20).

Results

Enhanced Growth Inhibition by Combinations of 17-AAG and Cytotoxic Agents. Taxol and doxorubicin are among the most active cytotoxic agents for the treatment of breast cancer. Exposure to these cytotoxic agents has been associated with the induction of Hsp90. Induction of Hsp90 may promote cell survival under these conditions (13, 20). Therefore, we evaluated whether modulation of Hsp90 function by 17-AAG leads to sensitization of cells to cytotoxic agents. The

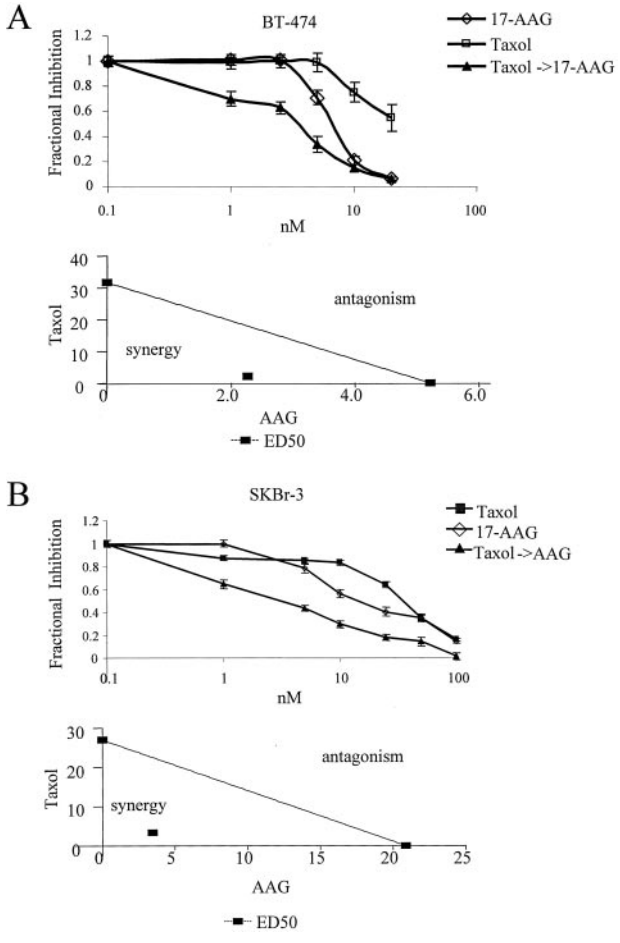

Fig. 1 17-AAG and Taxol-induced synergistic growth inhibition; cytotoxicity and isobolograms. The breast cancer cell lines BT-474 (*A*) and SKBr-3 (*B*) were exposed to either diluent or increasing concentrations of Taxol (0–100 nM) for 4 h, followed by either DMSO or continuous exposure to increasing doses of 17-AAG (0–100 nM) for 96 h [17-AAG alone (◇), Taxol alone (□), 17-AAG and Taxol (▲)]. Fractional inhibition of growth was assayed by Alamar Blue and graphed as percentage of untreated cells as described in "Materials and Methods." The combination of 17-AAG and Taxol had significant antiproliferative activity at concentrations showing minimal or no single agent activity. Isobologram analysis was performed for the 50% isodoses (ED50) according to Chou *et al.* (21); isobologram analysis shows synergy for both cell lines.

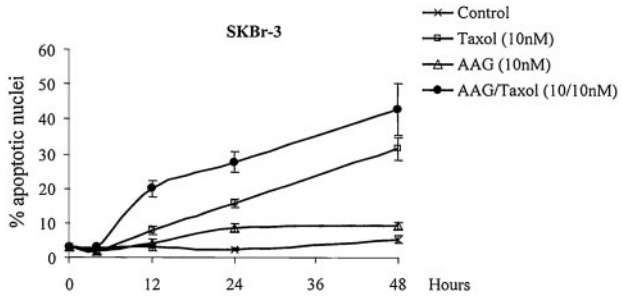

Fig. 2 17-AAG sensitizes cells to Taxol-induced apoptosis. Time course of induction of apoptosis in breast cancer cells treated with vehicle (×), 10 nM Taxol (□), 10 nM 17-AAG (△), or 10 nM Taxol and 17-AAG (●), as described in "Materials and Methods" and Fig. 1. Nuclei were assessed by quantitative fluorescence microscopy for apoptotic score at 0, 4, 12, 24, and 48 h and plotted in a line graph *versus* time. Two-hundred cells in five different fields were counted for each experiment. Each experiment was repeated in triplicate. *Error bars*, SE. Treatment with Taxol alone caused a linear increase in apoptosis (27.5 ± 3% *versus* 4 ± 0.5%; $P = 0.0004$) by 48 h. Exposure to 17-AAG alone caused an increase in apoptosis from 4 ± 0.5% to 9 ± 1% ($P = 0.008$) by 48 h. The addition of 17-AAG to Taxol resulted in a more pronounced increase in apoptosis compared with Taxol alone (42.5 ± 7.5% *versus* 27.5 ± 3%; $P = 0.017$). Enhanced apoptosis by the combinations was also seen at 12 and 24 h.

breast cancer cell lines SKBr-3 and BT-474 were treated with 17-AAG, Taxol, or both. Fractional inhibition of growth was determined by the Alamar Blue assay after 96 h. As shown in Fig. 1, *A* and *B*, both drugs caused growth inhibition. However, growth inhibition was more pronounced when the drugs were combined and occurred at concentrations at which each drug alone had little or no effect on cell proliferation. Mathematical analysis of the data by isobologram as described by Chou *et al.* (21) confirmed that the combination effects of Taxol and 17-AAG were synergistic (Fig. 1, *A* and *B*).

The Addition of 17-AAG-sensitized Cells to Apoptosis Induced by Taxol. Taxol causes stabilization of microtubules, arrest in mitosis, and apoptosis (22, 23). Therefore, we tested whether the synergistic effects of 17-AAG and Taxol as described above were attributable to an increase in Taxol-induced apoptosis. SKBr-3 cells were treated with 10 nM 17-AAG, 10 nM Taxol, or both and assessed at various time points. Apoptotic nuclei were scored by evaluating nuclear DNA fragmentation and condensation as described in "Materials and Methods." As shown in Fig. 2*A*, treatment with Taxol alone caused a linear increase in apoptosis as a function of time. The number of apoptotic nuclei increased from 4 to 27.5% by 48 h. Exposure of cells to 17-AAG alone caused a small increase in apoptosis, 4 to 9%. However, the addition of 17-AAG to Taxol resulted in a significant increase in apoptosis compared with Taxol alone (42.5% *versus* 27.5%). These findings were most pronounced at earlier time points (12 h), when neither agent had a significant effect.

The increase in apoptosis by 17-AAG combined with Taxol was also demonstrable by other assays frequently used to quantitate apoptosis, including measurement of NMPs and poly-(ADP-ribose) polymerase cleavage, as well as the activation of caspases 9 and 3. NMPs are insoluble components of the nuclear structural framework that are released and solubilized after cell death. Induction of NMP was evaluated in the supernatant of SKBr-3 cells treated with increasing concentrations of Taxol for 4 h followed by continuous exposure to either DMSO or 17-AAG. Supernatants of cells treated with Taxol (T: 1 nM, 10 nM, 50 nM) and 17-AAG (A: 50 nM) showed a significantly higher increase in NMP compared with those treated with Taxol alone at all of the indicated Taxol concentrations (T1 *versus* T1A50: 0% *versus* 28%, $P = 0.028$; T10 *versus* T10A50: 23% *versus* 96%, $P = 0.027$; T50 *versus* T50A50: 102% *versus* 145%, $P < 0.0001$).

An increase in the NMP by 17-AAG alone was not measurable at that time point. Enhancement of Taxol-induced apoptosis in SKBr-3 cells was also demonstrated when evaluating poly(ADP-ribose) polymerase cleavage (data not shown).

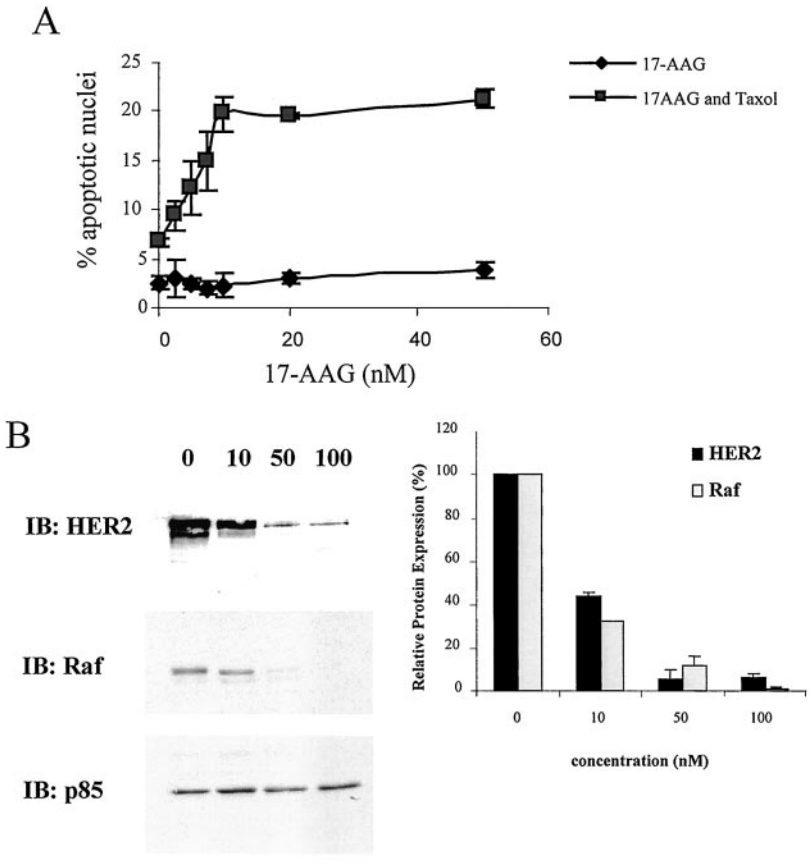

Fig. 3 Effects of 17-AAG on apoptosis and expression of target proteins. *A*, SKBr-3 cells were treated with vehicle (◆) or 10 nM Taxol (■) for 4 h followed by increasing concentrations of 17-AAG (0–50 nM). Apoptotic score was assessed at 12 h. Increasing concentrations of 17-AAG alone caused minimal increase in apoptosis. 17-AAG (0–10 nM) added to Taxol caused a steep increase in apoptosis (6.8% to 19.8%) and then reached a plateau. *B*, immunoblot of loss of HER2 and Raf in SKBr-3 cells treated with 0, 10, 50, and 100 nM of 17-AAG for 12 h. Samples of 50-μg NP40 lysates were loaded on each *lane*. Unaffected p85 expression served as control for equal protein loading. Protein expression of HER2 and Raf was decreased by 56% and 67% at 10 nM 17-AAG and over 90% at 50 nM.

Induction of Apoptosis Involves Activation of Caspases 9 and 3. Caspases are a family of cysteine proteases that play an integral role in apoptosis. Caspases can be separated into two groups, the initiators of cell-death signaling (caspases 8 and 9) and the effectors of cell disassembly (caspases 3, 6, and 7; Ref. 24). We studied the effects of 17-AAG and Taxol on one representative of each group. Caspase activation was measured in cell lysates of SKBr-3 cells at 12 and 24 h after exposure to 17-AAG (50 nM) and Taxol (10 nM). Compared with baseline, evaluation of activated caspase 9 levels at 24 h showed that treatment of SKBr-3 cells with the combination of 17-AAG and Taxol led to a significantly higher increase in activated caspase 9 levels (3.6-fold) than either drug alone (AAG, 1.5-fold; T, 2.2-fold). Similar results were seen for the increase in levels of activated caspase 3 (A, 1.9-fold; T, 2.2-fold; TA, 3.5-fold).

Effects of 17-AAG on Apoptosis. As seen in Fig. 2, dose-response curves for increasing doses of Taxol with or without 17-AAG were linear. The effects of 17-AAG on apoptosis were assessed in SKBr-3 cells using increasing doses of 17-AAG (0–50 nM) either alone or combined with 10 nM Taxol. Apoptosis was assessed 12 h after the addition of 17-AAG. As seen in Fig. 3*A*, increasing concentrations of 17-AAG alone caused a small increase in apoptosis. In contrast, adding up to 10 nM 17-AAG to Taxol caused a steep increase in the Taxol-induced apoptosis compared with 17-AAG alone. Doses higher than 10 nM caused no further increase. Ten nM 17-AAG was sufficient to cause more than 50% loss of expression of Raf and HER2 (Fig. 3*B*).

17-AAG Increases Taxol-induced Apoptosis in Multiple Breast Cancer Cell Lines. 17-AAG caused sensitization of SKBr-3 cells to Taxol as demonstrated by several assays of apoptosis. SKBR-3 cells are RB-positive breast cancer cells with high expression of HER2 and the absence of ER expression. We studied the effects of these two drugs in several other breast cancer cell lines with different characteristics: BT-474 (RB+, ER+, and high HER2), MDA-435 (RB+, ER−, and intermediate HER2), BT-549 (RB−, ER−, and intermediate HER2), MCF-7 (RB+, ER+, and low HER2), and MDA-468 (RB−, ER−, and nondetectable HER2). Apoptotic nuclei were scored at 12 h after Taxol treatment. Sensitization was observed in all of the examined breast cell lines independent of RB status, HER2, and ER expression; however, the concentrations of 17-AAG required to induce apoptosis and sensitization varied among different cell lines (BT-474, 10 nM; BT-549 and MDA-435, 50 nM; MCF-7, 100 nM; and MDA-468, 500 nM). Lower concentrations of 17-AAG were required in cells with higher levels of HER2 expression.

Sensitization of Cells to Taxanes by 17-AAG Is Schedule-dependent. Treatment of cancer cells with Taxol causes arrest in mitosis because of stabilization of microtubules (18). However, 17-AAG causes a RB-dependent growth arrest in G_1 (9). Thus, the G_1 arrest induced by 17-AAG in these cells may

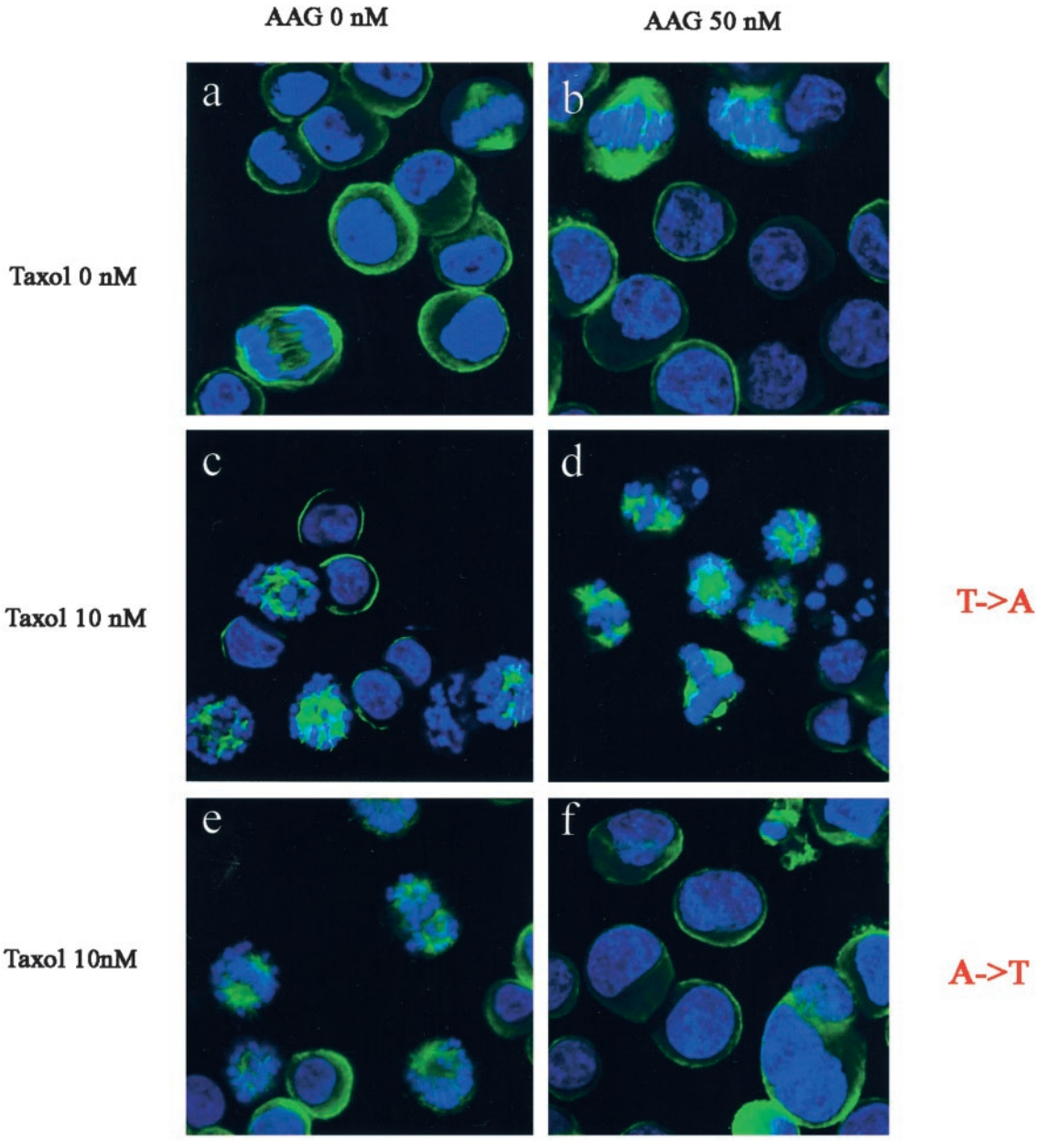

Fig. 4 Cell cycle arrest of drug combination is sequence-dependent in cells with positive RB. Confocal microscopy images of SKBr-3 cells treated with vehicle (*a*), Taxol 10 nM for 4 h (*c* and *e*), Taxol 10 nM for 4 h followed by 50 nM 17-AAG (*d*), or 24-h pretreatment with 50 nM 17-AAG followed by 10 nM Taxol (*f*). Cells were fixed with paraformaldehyde and stained with a fluorescent-labeled anti-β-tubulin and with Hoechst 33258 for nuclear DNA. The majority of DMSO- and 17-AAG-treated SKBr-3 cells were in interphase (*a* and *b*). Only a few cells were in mitosis. Mitotic figures showed normal tubulin staining with spindles emanating from two opposite poles. Treatment with Taxol caused an increase in the number of mitotic and apoptotic nuclei (*c*). Mitotic block occurred in metaphase/anaphase with irregular chromosomal arrangement and the formation of multiple asters (*c* and *e*). When 17-AAG was added after Taxol, the fraction of aberrant mitotic and apoptotic cells increased further (*d*). Accumulation of cells occurred in interphase with few apoptotic cells after pretreatment of 17-AAG (*f*).

prevent effects of the taxanes. We tested whether the sensitization of cells to Taxol by 17-AAG is schedule-dependent. SKBr-3 cells were treated with 10 nM Taxol or vehicle for 4 h. 17-AAG was added either 24 h before or 4 h after Taxol. As seen in Fig. 4 and Table 1, 93% of untreated and 92% of 17-AAG-treated SKBr-3 cells were in interphase (Fig. 4, *a* and *b*). Only a few cells were in mitosis (5% and 3%, respectively). Mitotic figures showed normal tubulin staining with spindles emanating from two opposite poles. In contrast, Taxol treatment resulted in a significant increase of cells in mitosis (17%) and, to a lesser degree, undergoing apoptosis (7%; Fig. 4*c*; Table 1). The Taxol-induced mitotic block occurred in metaphase/anaphase and was characterized by irregular chromosomal arrangement and the formation of multiple asters (Fig. 4*c*). When

Table 1 Assessment of cell cycle by fluorescent microscopy of SKBr-3 cells treated with DMSO, 10 nM Taxol for 4 h, 10 nM Taxol followed by 50 nM 17-AAG, or 24-h pretreatment with 50 nM 17-AAG followed by 10 nM Taxol

Cells were fixed with paraformaldehyde and stained with a fluorescent-labeled anti-β-tubulin and with Hoechst 33258 for nuclear DNA.

Drug	Interphase	Mitosis	Apoptosis
Untreated	93 ± 0.7	5 ± 0.7	2 ± 0.4
AAG 50 nM	92 ± 0.8	3 ± 0.6	5 ± 0.3
Paclitaxel 10 nM	76 ± 1.0	17 ± 1.0	7 ± 0.3
Paclitaxel → AAG	65 ± 1.2	13 ± 1.4	22 ± 1.0
AAG → Paclitaxel	89 ± 1.0	5 ± 0.8	6 ± 0.9

17-AAG was added after Taxol, the fraction of mitotic (13%) and apoptotic (22%) cells increased (Fig. 4*d*). In contrast, when 17-AAG was added before Taxol, most cells (89%) were in interphase, and only a few cells were apoptotic (6%; Fig. 4*f*). Further analysis showed that induction of apoptosis was most pronounced when 50 nM of 17-AAG was added simultaneously with Taxol (5 nM) or 4 h afterward. Sensitization was diminished by a 12-h pretreatment and almost completely inhibited by a 24-h pretreatment of 17-AAG (Fig. 5*A*). The inhibitory effects of 17-AAG pretreatment were more pronounced at higher doses of Taxol (Fig. 5*B*). Furthermore, pretreatment with 17-AAG also caused an inhibition of the Taxol-induced activation of caspase 9 (Fig. 5*C*). These data suggest 17-AAG sensitizes cells to Taxol-induced apoptosis in mitosis, whereas pretreatment of cells with 17-AAG results in G_1 block and prevents the Taxol effects.

Administration of 17-AAG and Taxol Is Not Schedule-dependent in RB-negative Cells. As described previously, in cells with mutated RB, 17-AAG causes growth arrest in mitosis and apoptosis. As shown in Fig. 5*D*, Taxol alone induced apoptosis in the RB-negative breast cancer cell line BT-549 in a linear manner. Enhancement of Taxol-induced apoptosis was observed independent of schedule. These results were confirmed in MDA-468, another RB-negative cell line. In these cells, 17-AAG and Taxol each caused growth-arrest mitosis. Our findings suggest that the addition of 17-AAG enhanced the mitotic arrest induced by Taxol irrespective of schedule. This was followed by apoptosis.

Sensitization of Cells to Doxorubicin Is Not Dependent on Schedule or RB Status. Doxorubicin is a DNA-intercalating agent that acts in all of the phases of the cell cycle (25). Doxorubicin-treated SKBr-3 (RB-positive) and BT-549 (RB-negative) cells accumulated in G_1 and G_2-M. Evaluation of nuclear DNA showed that cells were not arrested in mitosis. SKBr-3 and BT-549 cells were treated with doxorubicin (4 h) followed by 17-AAG or after a 24-h pre-exposure to 17-AAG. In both cell lines, pre-exposure to 17-AAG did not abrogate the sensitization but enhanced the apoptosis induced by doxorubicin. Doxorubicin-induced apoptosis was also enhanced when cells were exposed to 17-AAG immediately after doxorubicin (Fig. 6, *A* and *B*). The combination of doxorubicin and 17-AAG was synergistic in both cell lines.

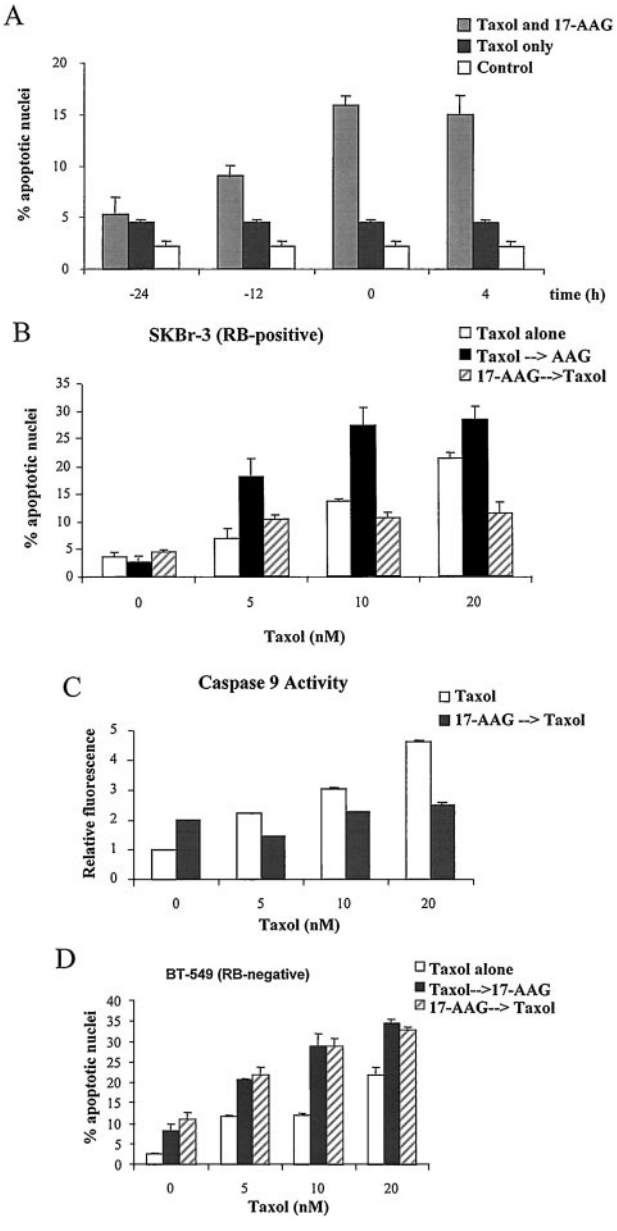

Fig. 5 Synergy between Taxol and 17-AAG is schedule-dependent in RB-positive but not RB-negative cells. *A*, SKBr-3 cells were treated with vehicle 5 nM Taxol and 50 nM 17-AAG at the indicated times before or after Taxol. Pretreatment with 17-AAG decreased the Taxol-induced apoptosis. This was more pronounced with 24-h than with 12-h pretreatment. *B*, SKBr-3 cells were treated with vehicle or Taxol (0, 5, 10, and 20 nM) for 4 h. 17-AAG (50 nM) was added 24 h before or immediately after Taxol. Apoptosis was assessed scoring apoptotic nuclei. *Error bars*, SE. Treatment with 50 nM 17-AAG before Taxol decreased (5 nM) or inhibited (24 h) apoptosis. *C*, caspase activity of caspase 9 measured by relative increase in fluorescence intensity of substrate; SKBr-3 cells were treated with 50 nM 17-AAG or DMSO for 24 h followed by 0, 5, 10, or 20 nM Taxol for 4 h. Bar graph of relative increase in fluorescence intensity measured at 12 h. *Error bars*, SE. *D*, BT-549 cells (RB-negative) were treated with Taxol (0, 5, 10, and 20 nM) for 4 h. 17-AAG (50 nM) was added 24 h before or immediately after Taxol. 17-AAG sensitized the Taxol-treated cells to apoptosis at all of the indicated concentrations ($P < 0.05$). Apoptosis was not dependent on sequence of 17-AAG administration ($P > 0.05$).

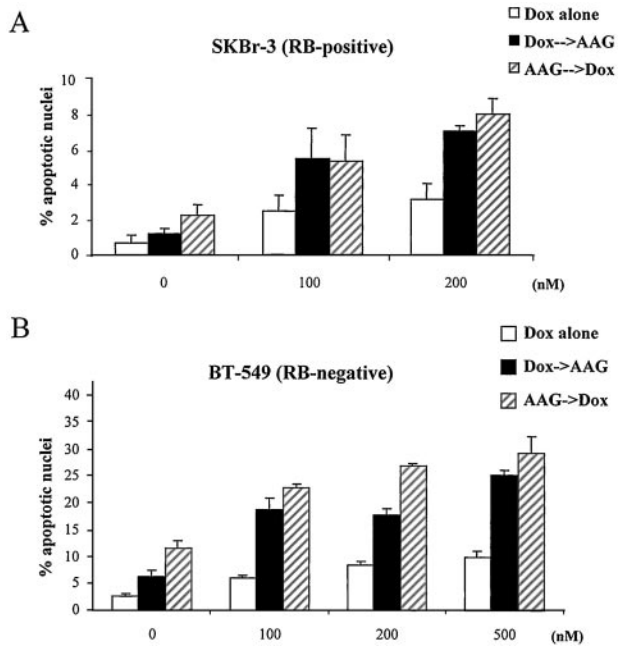

Fig. 6 Synergy between doxorubicin and 17-AAG is schedule-independent in cells with or without functional RB status. Apoptosis was assessed scoring apoptotic nuclei. Two-hundred cells in five different fields were counted for each experiment. Each experiment was repeated in triplicate. *Error bars*, SE. *P*s were determined by two-sided Student's *t* test. SKBr-3 (RB-positive) and BT-549 (RB-negative) cells were treated with doxorubicin (0 nM, 100 nM, 200 nM, and 500 nM) for 4 h. 17-AAG (50 nM) was added 24 h before (*AAG → Dox*) or immediately after (*Dox → AAG*) doxorubicin. The addition of 17-AAG significantly enhanced the apoptosis induced by doxorubicin in SKBr-3 cells (*A*; Dox 100, $P < 0.05$; Dox200, $P < 0.05$). Sensitization was independent of schedule (Dox100, $P = 0.42$; Dox200, $P = 0.39$). Schedule-independent sensitization was also seen in BT-549 cells (Dox 100, $P < 0.001$; Dox 200, $P = 0.004$; Dox 500, $P < 0.001$; *B*).

Discussion

Ansamycins are novel anticancer agents that inhibit Hsp90 by occupying its NH_2-terminal ATP-binding site (1, 2). Hsp90 is involved in both maturation of a number of key signaling proteins and the refolding of proteins in cells exposed to stress (3–8). The expression of Hsp90 is induced when cells are exposed to heat. However, a wide range of other environmental conditions has also been shown to induce Hsp90 expression, including exposure to radiation and cytotoxic agents. These findings suggest that Hsp90 may play an important role in survival of cells exposed to cytotoxic drugs, and the modulation of Hsp90 function by 17-AAG may render these cells more susceptible to therapeutic agents.

In this study, we report the effects of 17-AAG on cells exposed to cytotoxic agents. Taxol and doxorubicin induce apoptosis in breast cancer cell lines and are effective agents for the treatment of breast cancer. 17-AAG is the first of its class of Hsp90 inhibitors to be examined in clinical trials (11). Therefore, we evaluated whether modulation of Hsp90 by 17-AAG would enhance the apoptosis induced by these cytotoxic agents.

Our results show that 17-AAG enhanced the antiproliferative and antiapoptotic effects of Taxol or doxorubicin in several breast cancer cell lines. These effects were synergistic. When treated with Taxol, sensitization of cells to apoptosis was schedule- and RB-dependent. The effects were most pronounced when 17-AAG was given at the same time or immediately after Taxol. Treatment of cells with Taxol caused mitotic arrest and apoptosis. We found that pretreatment of RB-positive cells with 17-AAG led to an accumulation of cells arrested in G_1. This abrogated sensitization and, at longer pre-exposure, even inhibited the Taxol-induced apoptosis. Most of the cell lines that were examined for this study have doubling times that exceed 24 h. 17-AAG affects G_1 progression; it takes 12 to 24 h for cells to accumulate in G_1. This schedule dependence was also observed for activation of the caspases 3 and 9. We found that increasing concentrations of Taxol increased the activity of caspase 9 up to 5-fold. Treatment with 17-AAG alone increased caspase activity by 2-fold. In cells pretreated with 17-AAG, exposure to Taxol did not increase the caspase activity beyond 2-fold, suggesting that the Taxol-induced induction of caspase activity does not occur in cells arrested in G_1. We have reported previously that 17-AAG alone causes apoptosis. However, at early time points, the 17-AAG-induced apoptosis was minimal.

In cells with mutated RB, 17-AAG caused cell cycle arrest in mitosis, followed by apoptosis. In these cells, the 17-AAG-induced sensitization to Taxol was not schedule-dependent. It has been suggested that Raf activation by Taxol is required for Taxol-induced p21 activation and induction of apoptosis, whereas depletion of Raf by 17-AAG may lead to inhibition of both (26). We found that pretreatment of cells with 17-AAG led to a loss of Raf expression. However, in RB-negative cells 17-AAG enhanced Taxol-induced apoptosis despite Raf depletion. These data suggest that Raf depletion is not sufficient to abrogate Taxol-induced apoptosis.

We have shown that increasing concentrations of Taxol lead to a linear increase in apoptosis as a function of time and concentration. This was not the case for 17-AAG. At low concentrations, a steep increase in the enhancement of Taxol-induced apoptosis was noted. Concentrations higher than 10 nM, however, caused only minimal additional effects. Treatment of SKBr-3 cells with 10 nM 17-AAG caused a decrease in expression of HER2 and Raf by more than 50%. Loss of expression of these two proteins suggests an inhibition of Hsp90 function by 17-AAG at these concentrations. These results support the hypothesis that 17-AAG sensitized cells to Taxol-induced apoptosis by modulation of Hsp90 function.

In most of the experiments in this study, we evaluated the effects of 17-AAG on apoptosis rather than on growth arrest induced by cytotoxic agents. Although cell viability assays showed that in SKBr-3 and BT-474 cells combinations of 17-AAG and Taxol caused synergistic growth inhibition, synergy could only be evaluated at a concentration that caused minimal growth inhibition by each drug alone. Higher concentrations of each drug alone caused pronounced antiproliferative effects and may have masked additive or synergistic effects. Combination of the drugs at these concentrations may not show synergistic effects that stem from modulation of Hsp90 function.

Apoptosis can be quantitated by several assays, such as DNA fragmentation (27) and DNA laddering, annexin V staining, poly (ADP-ribose) polymerase cleavage, activation of caspases, and others (28–32). Many of these tests are dependent

on the cell type or the type of intervention; *e.g.,* MCF-7 cells lack caspase 3, thus some of the methods are not valid in this cell line (33). In this paper, we have quantitated apoptosis by different assays, but focused on scoring apoptosis analyzing DNA fragmentation with tubulin and bis-benzimide stains to distinguish between apoptosis, mitosis, and interphase cells. However, the results were confirmed using different quantitative apoptosis assays.

17-AAG enhanced the apoptosis induced by doxorubicin. Several investigators (25, 34, 35) have shown that the nature of the cell cycle arrest induced by doxorubicin may be p53-dependent. None of the cell lines used for these studies had functional p53. We found that cells treated with doxorubicin accumulated predominantly in G_1 and G_2 but not mitosis. As expected, sensitization of cells to doxorubicin by 17-AAG was not dependent on sequence or RB status.

HER kinases are among the most sensitive targets of ansamycins. Therefore, one might expect that 17-AAG may be effective in tumors that are dependent on HER2 for tumor growth and development, such as breast cancers that overexpress HER2. Preclinical and clinical data (36–39) have shown that additive or super-additive effects were seen when a monoclonal antibody directed against HER2 was combined with Taxol. The mechanism of these effects has not been clearly established. We have seen an enhancement of Taxol-induced apoptosis in several cell lines; however, cell lines with high HER2 expression such as BT-474 and SKBr-3 required lower concentration of 17-AAG to induce inhibition of cell proliferation and apoptosis either alone or in combination. Although these studies suggest that 17-AAG and Taxol or 17-AAG and doxorubicin may be most effective in cells with high expression of HER2, this has to be confirmed in additional studies.

The role of the heat shock protein chaperones in differentiation and apoptosis is not yet clearly established. However, several reports suggest their involvement in inhibiting apoptosis. It has been shown that Hsp90 binds to Apaf-1 and interferes with the cytochrome *c*-mediated activation of caspase 9 (40). The antiapoptotic effect of BAG has been associated with its binding to Hsp70 (41). Binding of Hsp90 to AKT kinase has been reported to maintain its kinase activity and to promote cell survival (42). We found that modulation of Hsp90 function by 17-AAG leads to activation of caspase 9 alone and when added to cytotoxic agents. This study suggests that the modulation of Hsp90 by 17-AAG significantly enhanced the apoptosis induced by the cytotoxic agents Taxol and doxorubicin. However, synergistic effects are dependent on the type of cell cycle arrest induced by the cytotoxic agent. If 17-AAG is administered with cytotoxic agents that cause cell cycle arrest in mitosis, such as the taxanes, the *Vinca* alkaloids, or epothilones, the sequence of these drugs may significantly impact their efficacy. On the other hand, it appears that the sequence of drug administration may be less crucial when 17-AAG is combined with anthracyclines that block in G_1 and G_2 or 5-fluorouracil that causes G_1 block. These findings are of critical importance for the design of clinical trials of 17-AAG in combination with cytotoxic agents.

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