Inhibition of Heat Shock Protein 90 Function by Ansamycins Causes the Morphological and Functional Differentiation of Breast Cancer Cells

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ABSTRACT

17-(Allylamino)-17-demethoxygeldanamycin (17-AAG) is an ansamycin antibiotic that binds to a conserved pocket in Hsp90 and induces the degradation of proteins that require this chaperone for conformational maturation. 17-AAG causes a retinoblastoma (RB)-dependent G1 block in cancer cells and is now in clinical trial. In breast cancer cells, G1 block is accompanied by differentiation and followed by apoptosis. The differentiation is characterized by specific changes in morphology and induction of milk fat proteins and lipid droplets. In cells lacking RB, neither G1 arrest nor differentiation occurs; instead, they undergo apoptosis in mitosis. Introduction of RB into these cells restores the differentiation response to 17-AAG. Inhibitors of the ras, mitogen-activated protein kinase, and phosphatidylinositol 3-kinase pathways cause accumulation of milk fat proteins and induction of lipid droplets when associated with G1 arrest but do not cause morphological changes. Thus, regulation of Hsp90 function by 17-AAG in breast cancer cells induces RB-dependent morphological and functional mammary differentiation. G1 arrest is sufficient for some but not all aspects of the phenotype. Induction of differentiation may be responsible for some of the antitumor effects of this drug.

INTRODUCTION

The ansamycin antibiotics HA and GM bind to a conserved pocket in the chaperone Hsp90 (1). This pocket has homology to the ATP-binding site of DNA gyrase and has low affinity for ATP and ADP (2). Occupancy of this pocket by the ansamycins prevents refolding of mature proteins and causes the degradation of several key signaling proteins (3, 4). These include Raf serine kinase, mit tyrosine kinase, steroid receptors, and members of the HER kinase family (5–10). The mechanism of this effect is complex. In some systems, GM has been shown to prevent the ATP-dependent release from Hsp90 of the protein undergoing refolding (4). The stabilized complex is then ubiquitinated and degraded (6, 9, 11, 12). This leads to degradation rather than maturation of target proteins. When added to cells, ansamycins cause the proteosomal degradation of the subset of cellular proteins that is dependent on this chaperone and the down-regulation of the signaling pathways controlled by these proteins (6, 9, 11, 13–15). Among the most sensitive targets are the HER kinases, including HER2 (9, 16, 17). As predicted, this leads to growth arrest and cell death. These drugs have potent antitumor activity and a derivative of GM, 17-AAG, is currently in clinical trial.

The importance and the number of the target proteins affected by these drugs suggest that they might cause nonspecific cell death by deranging multiple cellular housekeeping functions. However, the cellular effects of ansamycins are rather specific. GM and HA cause an RB-dependent G1 block that is mediated by selective down-regulation of pathways responsible for induction of cyclin D-cyclin-dependent kinases 4,6 protein kinase activity (18, 19). G1 progression in cells with mutated RB is unaffected by GM and HA. Furthermore, addition of 17-AAG to tumor-bearing animals has antitumor activity at nontoxic doses (National Cancer Institute monograph). In fact, tumor cells dependent on HER2 are very sensitive to these drugs. These data and the initiation of clinical trials of this drug in advanced cancer patients led us to further investigate the effects of 17-AAG on breast cancer.

We found that 17-AAG inhibits the proliferation of breast cancer cell lines by causing them to arrest in the G1 phase of the cell cycle. G1 arrest is RB dependent and followed by the induction of mammary differentiation. Subsequently, these cells undergo apoptosis. Differentiation is characterized by the appearance of fat-containing vacuoles, induction of milk fat proteins, and other morphological changes characteristic of normal mammary epithelia. Induction of growth arrest with nutrient deprivation or inhibitors of the ras, MAP kinase, and PI3 kinase pathways results in biochemical, but not morphological, differentiation. Arrest of growth in G1 is necessary but not sufficient for induction of the full phenotype. Cells lacking RB function arrest in mitosis when exposed to 17-AAG and undergo apoptosis but do not differentiate. These results suggest that regulation of Hsp90 by 17-AAG reverses the dedifferentiation characteristic of mammary carcinoma, and this effect may play a role in the antitumor activity of the drug.

MATERIALS AND METHODS

Chemicals. GM and 17-AAG were kindly supplied by Dr. E. Sausville (National Cancer Institute, Bethesda, MD) and dissolved in DMSO. LY294002 and rapamycin were purchased from Sigma Chemical Co.; PD98059 (a specific inhibitor of MEK) from Parke Davis; L744832 from Merck; and FK-011 (BOC) caspase inhibitor was purchased from Enzyme System Products.

Antibodies. Anti-casein was from Harlan Sera (Indianapolis, IN), and antibodies against the human milk fat globulin protein (polyclonal) and MFGM protein (monoclonal) were from Chemicon. Polyclonal antibodies against PARP and RB were from Santa Cruz Biotechnology. Monoclonal antibody against β-tubulin, ethidium bromide, and acridine orange were purchased from Sigma; bis-benzamide trihydrochloride (Hoechst #33258; DAPI) from Hoechst. Oil Red O staining kit was purchased from Poly Scientific Chemicals. Nile Red powder [9-diethylamino-5H-benzo(a)phenoxazine-5-one] was purchased from Sigma and dissolved in acetone at a stock solution of 1 mg/ml. All other reagents were of analytical grade and purchased from standard suppliers.

Cell Culture. The human breast cancer cell lines SKBr-3, MDA-MB-231 (MDA-231), MCF-7, BT-474, BT-549, and MDA-MB-468 (MDA-468) were from the American Type Culture Collection (Rockville, MD). Cell lines were maintained in medium consisting of DMEM/F12 (1:1) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 50 units/ml of both penicillin and streptomycin in a humidified 5% CO2/air atmosphere at 37°C. For RB transfection, MDA-468 cells were transfected with the plasmid pHUH10-[3H]GR containing full-length, 4.7-κb human RB cDNA. RB trans-
fectants were grown in DMEM/F12 media supplemented with 5% FCS (Life Technologies, Inc.), 2 mM glutamine, and 50 μg/ml each of penicillin and streptomycin and 100 μg/ml hygromycin B (Boehringer Mannheim).

Antiproliferative Index. Cells (2 × 10^{4}) were plated onto six-well dishes and treated with the indicated drug or DMSO vehicle for 96 h. Drug and media were exchanged every 48 h. After 96 h, medium was removed, and cells were washed with PBS and harvested. Cells were counted on a Coulter counter. Dose curves were plotted as a logarithmic function of cell number versus concentrations.

Drug Treatment of Cell Lines. Cells were plated onto 100-mm tissue culture plates at a density of 2 × 10^{9} for 48 h and then treated with 17-AAG or equal concentrations of the vehicle. For longer drug exposure times, medium with drug or vehicle were exchanged every 48 h. When using a caspase inhibitor, drug was changed every 24 h attributable to its short half-life.

Detection of Apoptosis and Mitosis. Apoptosis was scored by the presence of nuclear chromatin condensation and DNA fragmentation and evaluated with fluorescence microscopy. Mitosis was scored by typical DNA condensation and formation of tubulin structures emanating from two opposing poles. Cells were harvested and fixed in 100% ethanol at −20°C for 10 min. After washing twice with PBS, cells were incubated with anti-β-tubulin primary and fluorescently labeled secondary antibody. Nuclei were stained with 2 μg/ml of the dye bis-benzimide trihydrochloride (Hoechst #33258). Two hundred cells were counted for each experiment in five different fields and evaluated for apoptotic and mitotic scores (apoptotic or mitotic nuclei/all nuclei × 100%).

Each experiment was repeated in triplicate. SE denotes error of the mean.

Cell Cycle Analysis. Cell cycle distribution was assayed according to Nusse et al. (20) with a Becton Dickinson fluorescence-activated cell sorter and analyzed by Cell Cycle Multi-Cycle system (Phoenix Flow System, San Diego, CA).

Quantitative and Qualitative Assessment of Differentiation. Oil Red O staining was performed according to the protocol described by Poly Scientific. In brief, cells were fixed with a quick dip in 100% methanol and washed in distilled water. Slides were then incubated with Oil Red O stain for 30 min at room temperature. After incubation, slides were differentiated with an 85% propylene glycol solution for 1 min, rinsed in water, and stained in Mayer’s Hematoxylin. For Nile Red staining, we used a protocol described by Green et al. (24). In brief, live cells were washed with HEPES buffer and resuspended in a 1:200 dilution of Nile Red in HEPES buffer (stock solution 1 mg/ml Nile Red in acetone) for 5 min. Fluorescent intensity was assessed in the green, orange, and red spectra with a Becton Dickinson fluorescence analyzer. Fluorescent intensity was compared with that of untreated cells. Nonpolar or neutral lipids stained bright green-yellow; polar lipids stained dark red when assessed with a broad band green fluorescent protein filter. For quantitative analysis, the number of treated cells with increased intensity in fluorescence was compared with that of untreated cells. The fluorescent intensity seen in the 5% of untreated cells with the strongest fluorescent signal and was used as an arbitrary baseline. The percentage of treated cells exhibiting equal or greater fluorescent intensity was then scored.

H&E Stain. Cell monolayers on slides were fixed with paraformaldehyde (4%) for 10 min at room temperature and stained according to standard H&E staining protocols (22).

Immunoblotting and Immunofluorescence. For immunoblotting, cells were harvested in medium, washed twice in PBS, and then dissolved in NP40 lysis buffer (50 mM Tris-CI (pH 7.4), 1% NP40, 40 mM NaF, 150 mM NaCl, 10 mM/m of each Na_{2}VO_{4}, phenylmethylsulfonyl fluoride, and DTT, and 1 μg/ml each of leupeptin, aprotinin, and soybean trypsin inhibitor). Fifty μg of lysate were loaded onto SDS-PAGE mini-gels. As described previously, proteins were transferred to nitrocellulose membranes and incubated with primary and secondary antibodies. Proteins were visualized by chemiluminescence (ECL; Amersham Corp.) on Bio-Max film (Eastman Kodak Ref. 13).

For immunofluorescence, 10^2 cells were plated onto fibronectin-coated Lab-Tek two-well chamber slides (Fisher Scientific). After the experimental procedure, slides were washed twice with ice-cold PBS and fixed with methanol and acetone solution (1:1) for 15 s. Fixed monolayers were then washed with distilled water and blocked with 5% BSA in PBS solution. After blocking, cells were incubated with the primary antibody (1:100 in 5% BSA in PBS) at 37°C and washed three times with 1% BSA in PBS. Then, monolayers were incubated with fluorescein- and rhodamine-conjugated secondary antibodies for 1 h at 37°C. Nuclei were stained with DAPI at concentration of 1 μg/ml.

RESULTS

GM and 17-AAG Cause RB-dependent G_{1} Arrest Associated with Hypophosphorylation of RB. Previous studies have shown that HA and GM cause RB-dependent G_{1} block. G_{1} progression is unaffected by these drugs in cells with defective RB function; instead, they block in mitosis. We repeated these studies with 17-AAG, the agent currently undergoing clinical trial, and determined its potency in a panel of breast cancer cell lines. The lowest concentration that caused complete growth arrest was determined for each cell line. The sensitivity of cell lines varied from 10 to 1000 nM. As reported previously, cell lines with HER2 overexpression were especially sensitive to the drug (23). The effects observed on cell cycle cycle and differentiation occurred at the concentration of drug that caused growth arrest in that particular cell line and were not a function of the absolute concentration required. Because the doubling times of the tested cell lines varied from 18 to 48 h, the cell cycle effects of the drug were observed at 48 h. In the cell lines that contain intact RB (MCF-7, SKBr-3, and MDA-231), induction of growth arrest by 17-AAG was associated with accumulation of cells in G_{1} and loss of cells in S-phase. RB accumulated in the hypophosphorylated form (Fig. 1). This 17-AAG-induced G_{1} arrest was observed in seven additional tumor cell lines. An increase in cells with 4N DNA content was also observed in certain cell lines. This was lost upon longer exposure to drug. Assessment of nuclear DNA with DAPI stain showed that the amount of cells in mitosis decreased from 6% to <0.5% of the cell population after drug treatment (DNS). Furthermore, when these cell lines were arrested in G_{1}-S with aphidicolin and then released into assamycin, they progressed through mitosis and arrested in G_{1} (DNS). In contrast, in the breast cancer cell lines with mutated RB (MDA-468 and BT-549), 17-AAG caused an accumulation of cells in the G_{1}-M phase (Fig. 1). Because mitotic arrest is often transient, we evaluated cell cycle by nuclear DNA and anti-β-tubulin staining to distinguish between cells in interphase, mitosis, and apoptosis. We found that after a 24-h drug exposure, 35% + 3 of treated cells were mitotic and 5% + 0.6 apoptotic. After 48 h of drug exposure, the apoptotic index had increased to 26% ± 3, whereas the mitotic index was 22% ± 2. Mitotic and apoptotic indices of untreated cells were 4% ± 0.3 and 2% + 0.3, respectively (see Table 1). Treatment of a synchronized population of MDA-468 cells caused a mitotic index of 60–80% by 24 h. This occurred prior to induction of significant apoptosis but was subsequently followed by apoptosis.6

17-AAG Causes Apoptosis, and Apoptosis Is Not RB Dependent. To determine whether growth inhibition by 17-AAG is attributable to inhibition of cell proliferation alone or a combination of growth arrest and induction of cell death, we assessed whether 17-AAG caused apoptosis. We evaluated the increase in percentage of apoptotic nuclei in breast cancer cells treated with 17-AAG by staining them with DAPI. Over time, treatment with 17-AAG resulted in a significant increase in the number of apoptotic nuclei in the examined breast cancer cells (SKBr-3, MDA-231, MCF-7, and MDA-468; Fig. 2A). In the RB-positive cell lines requiring lower concentrations for complete growth arrest, such as SKBr-3, apoptosis was seen earlier and was more pronounced (Fig. 2A). This observation was also noted in three other cell lines, e.g., the breast cancer cell lines BT-474 and MDA-435 and the ovarian cell line, SKOV-3. All of these cell lines show elevated levels of HER2 expression. Similarly, significant apoptosis occurred early in the RB-negative cells; however, as described above, cells underwent apoptosis in mitosis.

Apoptosis was further assessed by evaluation of PARP cleavage

\[ 6 M. Srethapakdi, M. M. Mousse, and N. Rosen. The retinoblastoma protein is required for completion of mitosis when Hsp90 is inhibited, submitted for publication. \]
Cleavage of PARP requires the involvement of caspases, in particular, caspase-3 (24). Treatment of the cell lines SKBr-3, MDA-231, and MDA-468 caused cleavage of PARP from a Mr 116,000 protein into a characteristic Mr 85,000 fragment. At 48 h, significant PARP cleavage was seen in SKBr-3 and MDA-468 but not in MDA-231 and MCF-7. Appearance of an Mr 85,000 fragment was not observed in MCF-7 cells, even at time points when significant apoptosis was present (Fig. 2B). These cells lack the required caspase-3 (24). However, as demonstrated in Fig. 2A, 17-AAG caused apoptosis in MCF-7 cells, suggesting that it can activate caspase-3-dependent and -independent pathways. Apoptosis also occurred when RB-negative MDA-468 cells were treated with 17-AAG. The pattern of PARP cleavage was similar in cells with intact or mutated RB. However, in the RB-negative cells, apoptosis occurred in mitosis.

Induction of Morphological Differentiation by 17-AAG. Prior to the induction of apoptosis by 17-AAG, significant changes in morphology were noted in each of the cell lines with wild-type RB. In Fig. 3, we describe the morphological changes of MCF-7 cells. MCF-7 cells were derived from a metastatic ductal carcinoma. They were round cells with scanty eosinophilic cytoplasm and indistinct cell margins. The cells contained large, basophilic ovoid nuclei with the prominent nucleoli characteristic of carcinoma cells. They expressed minimal levels of milk fat proteins and contained few fat-containing vacuoles or other features of mammary differentiation. Considerable mitotic activity was present (Fig. 3, a–c).

In contrast, cells exposed to 17-AAG flattened, increased in size, became columnar in shape, and had distinct cellular boundaries. The increase in cell size was predominantly attributable to an abundance of cytoplasm, which led to a decrease in the nuclear:cytoplasmic ratio. The nuclei were round with sharp borders and had much less prominent nucleoli. There were almost no mitotic figures. Treated cells (Fig. 2B). Cleavage of PARP requires the involvement of caspases, in particular, caspase-3 (24). Treatment of the cell lines SKBr-3, MDA-231, and MDA-468 caused cleavage of PARP from a Mr 116,000 protein into a characteristic Mr 85,000 fragment. At 48 h, significant PARP cleavage was seen in SKBr-3 and MDA-468 but not in MDA-231 and MCF-7. Appearance of an Mr 85,000 fragment was not observed in MCF-7 cells, even at time points when significant apoptosis was present (Fig. 2B). These cells lack the required caspase-3 (24). However, as demonstrated in Fig. 2A, 17-AAG caused apoptosis in MCF-7 cells, suggesting that it can activate caspase-3-dependent and -independent pathways. Apoptosis also occurred when RB-negative MDA-468 cells were treated with 17-AAG. The pattern of PARP cleavage was similar in cells with intact or mutated RB. However, in the RB-negative cells, apoptosis occurred in mitosis.

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Table 1. Assessment of apoptosis and mitosis by nuclear DNA and anti-β-tubulin staining in 17-AAG treated MDA-468 cells using fluorescence microscopy

<table>
<thead>
<tr>
<th>Cell cycle/drug exposure</th>
<th>Interphase</th>
<th>Mitosis</th>
<th>Apoptosis</th>
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<tbody>
<tr>
<td>0 h</td>
<td>89% ± 1</td>
<td>4% ± 3</td>
<td>2% ± 0.3</td>
</tr>
<tr>
<td>24 h</td>
<td>61% ± 2</td>
<td>35% ± 3</td>
<td>5% ± 0.6</td>
</tr>
<tr>
<td>48 h</td>
<td>58% ± 2</td>
<td>22% ± 2</td>
<td>26% ± 3</td>
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* ± SE.
became more epithelial in appearance, with intercellular digitations and bridges. Many cells contained perinuclear vacuoles; however, these were more evident when using special stains (see below and Figs. 4 and 5). These morphological changes are characteristic of mature epithelial differentiation and reversal of transformation.

In MCF-7, the morphological changes began at 24 h after drug treatment, with induction of increasing numbers of intracellular lipid droplets. At that time, only minimal flattening and enlarging of cells was evident, but this became clearly visible by 48 h. The morphological changes were still present up to 120 h after drug addition, when apoptosis became predominant. Similar morphological changes were observed in SKBr-3 and MDA-231 cells (DNS). These findings were noted at the concentrations of drug that inhibited cell proliferation. Morphological changes were not observed in RB-negative cells, MDA-468 and BT-549.

**Induction of Lipid Droplets.** A specific secretory function of the differentiated mammary gland is the production of milk. Fat globules containing a core of triglycerides are one of the major components of human milk. The milk fat globule is enveloped by a membrane that consists of phospholipids, proteins, and glycoproteins. Breast cancer cell lines have very few detectable lipid vacuoles. As seen in Fig. 3, perinuclear vacuoles accumulated in MCF-7 cells exposed to 17-AAG. Staining of these cells with Oil Red O showed that these vacuoles were lipid droplets and located in the cytoplasm around the nucleus (Fig. 4A). Triglycerides are neutral lipids. Nile Red dye is a vital stain that distinguishes the components of intracellular lipid droplets. It is strongly fluorescent but only in a hydrophobic environment. Polar lipids, such as phospholipids, fluoresce dark orange in the presence of Nile Red, whereas neutral lipids fluoresce brilliant yellow-gold. The lipid droplets located in the perinuclear area fluoresced brilliant yellow-gold, suggesting that these droplets contained neutral lipids and may be milk fat triglycerides (Fig. 4B). As expected the cell membranes, which contain phospholipids, appeared dark orange. The milk fat globule membrane was not detected with this stain. Pretreatment of cells with isopropyl alcohol prior to Nile Red staining in vehicle or 17-AAG-treated cells extracted the bright green-yellow neutral fat droplets but not the dark orange staining polar lipids from the cell membranes. We can therefore conclude that neutral lipids are not bound to the cytoskeleton accumulated in the cytoplasm (Fig. 4B). These data suggest that 17-AAG induced triglyceride fat globules, a component of milk.

In contrast to other lipid stains such as Oil Red O and Sudan Black, Nile Red stain fluorescence can be quantitated. As seen in Fig. 4C,
treatment with 17-AAG caused an induction of neutral lipid in SKBr-3 by 24 h with a maximal increase by 48 h and a subsequent decline. Induction in MCF-7 followed similar kinetics, but the peak was delayed (Fig. 4C). The intensity of polar lipids/cell was also increased but to a lesser extent. This is consistent with the observed increase in cell size. Treatment of the RB-negative cell lines MDA-468 (Fig. 4C) and BT-549 with 17-AAG did not cause lipid induction.

**Induction of Milk Fat Proteins by 17-AAG.** Milk is a complex mixture of fat and proteins. In breast cells, milk fat is surrounded by a complex structure, the MFGM. Other components of milk are the milk fat globulins. The expression of a component of this membrane, the MFGM protein and that of milk fat globulin, was assessed in breast cancer cells exposed to 17-AAG. Untreated cell lines (MCF-7 and SKBr3) express basal levels of both proteins, as measured by Western blot and immunofluorescence (Fig. 5). Immunofluorescence analysis (Fig. 5A) revealed low levels of milk fat globulin expression in a perinuclear distribution in MCF cells and little expression in SKBr-3. Low background levels of MFGM protein were observed in both cell lines.

17-AAG induced the expression of these proteins in both cell lines (Fig. 5) by 24 h. After 72 h, the protein expression declined. Induction was concurrent with that of neutral lipid (Fig. 4C). After longer exposure, expression of these proteins declined. Milk fat globulin is localized in a clumped perinuclear distribution similar to that of the neutral lipids. In contrast, MFGM is distributed throughout the cytoplasm in a membranous pattern. This is more marked in MCF-7 than in SKBr3 cells (Fig. 5A). Furthermore, throughout the cytoplasm, empty vacuoles were noted that are surrounded by MFGM proteins. It is likely that these vacuoles contained the lipids that were extracted when the cells were fixed with methanol. The induction of casein was not detected in any of the examined cell lines. At 48 h, MFGM protein was detected easily in the culture medium of treated MCF-7 and SKBR-3 cells (DNS). Whether this is because of leakage of protein from dying cells or because of secretion of milk fat proteins has not been determined. The coinduction of milk fat globulin, MFGM protein, and neutral lipid suggests that ansamycins caused functional as well as morphological differentiation.

**Differentiation Induced by Ansamycins Is RB Dependent.** We examined the effects of 17-AAG on MDA-468, a breast cancer cell line with mutated RB. 17-AAG caused the growth arrest of this cell in mitosis but did not induce milk fat proteins or the accumulation of lipid droplets (Fig. 6). No changes in morphology were observed. In contrast, in MDA-468 that were stably transfected with RB, 17-AAG
caused an increase of the population of cells that were arrested in G₁. G₁ arrest occurred at concentrations that caused arrest in mitosis in the parental MDA-468. In these cells, 17-AAG caused a 2-fold increase in MFGM protein expression (Fig. 6A). Although the kinetics of the protein induction was similar to those in the RB-positive cell lines, the increase of total protein was less. A 2.5-fold induction of neutral lipid occurred, a less marked effect than that seen in the RB-positive cells (Fig. 6B). Furthermore, treatment of MDA-468 with the histone deacetylase inhibitor suberanilohydroxamic acid, an agent that is known to induce differentiation in other cell lines (25), MDA-468 caused cell cycle arrest in G₁, lipid induction, and morphological differentiation. These data suggest that G₁ arrest is required for induction of differentiation. Furthermore, differentiation induced by 17-AAG is RB dependent.

Growth Arrest Is Sufficient for Induction of Some but not All Aspects of the Differentiated Phenotype. Ansamycins cause the interruption of several signaling pathways and growth arrest in the G₁ phase of the cell cycle. Therefore, we asked whether any features of differentiation induced by 17-AAG could also be elicited by blocking proliferation in G₁ or with specific signaling inhibitors. SKBr-3 and MCF-7 cells were exposed to serum starvation or to a PI3 kinase inhibitor (LY294002), a MEK inhibitor (PD98059), a farnesyltransferase inhibitor (L744832), or rapamycin (a modulator of mTOR and S6 kinase).

Growth arrest was found to be sufficient for a more than 3-fold increase in expression of MFGM protein. However, lipid induction was only seen in association with G₁ arrest, and only 17-AAG caused significant changes in the morphology as described in Fig. 3 (Table 2). Inhibition of both the PI3 kinase and the MAP kinase pathways simultaneously did not induce the complete phenotype. Evaluation of iso-inhibitory concentration of currently used standard chemotherapy agents, such as doxorubicin, taxanes, and cisplatin, caused apoptosis but did not induce differentiation.

Fig. 4. Accumulation of cytoplasmic lipid droplets. A, light microscopy (×40) of MCF-7 cells treated with vehicle or 17-AAG (500 nM) for 48 h stained with Oil Red O. Treated cells showed an increase in intracytoplasmic lipid droplets. B, immuno-fluorescence microscopy of 17-AAG-treated SKBr-3 (50 nM) and MCF-7 (500 nM) cells stained with Nile Red showed an accumulation of yellow-green fluorescing (neutral) lipids in the perinuclear area. The polar lipids of the membranes fluoresced in dark orange. Exposure to 100% isopropyl alcohol for 10 min prior to Nile Red staining eliminated the yellow fluorescing perinuclear lipid droplets in sections that were treated with vehicle (MCF-7) or with 17-AAG (SKBr-3). C, quantitative analysis of lipid induction. Cells were treated with 17-AAG (SKBr-3, 50 nM; MCF-7, 500 nM; MDA-468, 500 nM) and stained with Nile Red. Cells with increased fluorescent intensity were scored by fluorescence-activated cell sorter as described in “Material and Methods.” A column histogram of percentage of cells with increased fluorescent intensity is shown.
The Differentiated Phenotype Is Not a Stress Response to Apoptosis. Under conditions of environmental stress, cells undergo morphological change and vacuolization. Exposure of SKBr-3 cells to 100 nm 17-AAG for 48 h causes an increase in apoptosis with 18% of the cells containing apoptotic nuclei as opposed to 4% in the control. To determine whether induction of differentiation by 17-AAG was an early response to induction of apoptosis by a toxic drug, we used a pan-caspase inhibitor to prevent apoptosis. Cells were treated simultaneously with 17-AAG and BOC. Treatment with 1–100 μM BOC for 48 h did not affect cell growth and did not affect the number of apoptotic nuclei in untreated SKBR-3 cells. However, when SKBr-3 cells were treated with 100 μM BOC and 100 nm 17-AAG, complete growth arrest with cell cycle arrest in G₁ was seen by 48 h, but only 6% of cells were apoptotic. Despite a decrease in apoptosis, induction of lipids and changes in morphology still occurred and to the same extent in cells treated with 17-AAG and BOC. The caspase inhibitor alone did not cause lipid induction or morphological changes. These findings suggest that caspase activation is not necessary for induction of differentiation, and lipid induction is not likely to be an early marker of a toxic insult to the cell.

**DISCUSSION**

Ansamycin antibiotics are drugs that bind to a specific pocket in the Hsp90 family of chaperones and alter their function. Addition of these drugs to cells leads to the degradation of several important proteins that are involved in intracellular signaling (5–10, 12, 17).

Therefore, one might predict that these drugs would cause nonspecific cell death. On the contrary, in this paper, we report that 17-AAG, an ansamycin antibiotic now in clinical trial, causes breast cancer cells to arrest in G₁, undergo subsequent mammary differentiation, and then apoptosis.

Cells treated with 17-AAG undergo an RB-dependent cell cycle arrest in G₁. In cells with intact RB, the growth arrest is accompanied by morphological changes suggestive of reversion of transformation and differentiation to a more epithelial phenotype. The cells flatten and enlarge, the nuclear:cytoplasmic ratio decreases, and the number of nucleoli decline. Furthermore, drug treatment causes accumulation of perinuclear fat vacuoles, the development of MFGMs, and the induction of milk proteins, suggesting not only a morphological but also functional differentiation of these mammary cells. However, we could not determine whether these cells could further differentiate and form ducts or lobules upon longer drug exposure. This was in part because of the apoptosis and subsequent cell loss that followed differentiation.

It is possible that the differentiation of these cells is an obligatory response to growth arrest. Indeed, we find that a variety of manipulations that cause growth arrest, including serum starvation and pharmacological inhibition of the ras, P13 kinase, and MAP kinase pathways cause the induction of milk fat proteins. However, only those interventions that lead to arrest in G₁ cause significant accumulation of lipid droplets, and only 17-AAG causes the most differentiated phenotype, enzyme induction, lipid droplet accumulation, and morphological change. Furthermore, simultaneous inhibition of MAP kinase and P13 kinase signaling does not result in morphological differentiation. Ansamycins fail to induce G₁ arrest or differentiation in cells deficient in RB function. RB-negative cells treated with 17-AAG undergo mitotic arrest and apoptosis. In addition, when RB was restored to these cells, induction of lipid droplets and milk fat proteins by 17-AAG was observed.

From these data, it is not clear whether RB functions other than those required for G₁ arrest are necessary for induction of differentiation by ansamycins. We have shown the histone deacetylase inhibitor, suberanilohydroxamic acid, induces both G₁ arrest and morphological and functional differentiation in an RB-independent manner (DNS). Therefore, it is likely that the role of RB is confined to mediating G₁ arrest. As implied by these data, ansamycins do not affect histone acetylation (DNS) and therefore probably do not cause differentiation by affecting histone deacetylation.

G₁ arrest is necessary but not sufficient for morphological differentiation. In SKBr3 cells, ansamycins cause degradation of HER kinases with attendant down-regulation of P13 kinase and Ras signaling (data not shown), but P13 kinase and MEK inhibitors, alone or in combination, induced only part of the differentiated phenotype. Destruction of HER2 leads to down-regulation of other signaling pathways and in itself may be sufficient for induction of complete differentiation. In fact, anti-HER2 antibodies have been shown to induce morphological and functional differentiation. In breast cancer cells with high HER2, neu differentiation factor (heregulin) causes decreased HER2 expression and morphological and functional differentiation (26). It is possible that one consequence of HER2 overexpression in breast cancer is dedifferentiation (27).

### Table 2 Evaluation of differentiation markers induced by other signal transduction inhibitors

<table>
<thead>
<tr>
<th>Intervention</th>
<th>SKBr-3 MFGM protein (X-fold)</th>
<th>Specific cell cycle arrest</th>
<th>Lipid induction (X-fold)</th>
<th>Morphological differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17-AAG</td>
<td>1.0</td>
<td>G1 5.5</td>
<td>6.8</td>
<td>Present</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>+</td>
<td>G1 3.8</td>
<td>1.1</td>
<td></td>
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<tr>
<td>Serum starvation</td>
<td>+</td>
<td>G1 3.8</td>
<td>1.1</td>
<td></td>
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<tr>
<td>LY294002</td>
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SKBr-3 cells were treated with the indicated for interventions for 48 h and analyzed.

LY294002 (50 μM), P13 kinase inhibitor; PD 98059 (50 μM), MEK inhibitor; L744832 (20 μM), farnesyltransferase inhibitor 50.
Alternatively, induction of differentiation by ansamycins may involve HER kinase-independent pathways as well. We observed that ansamycins cause mammary differentiation in cells with both high and low levels of HER-2 expression. However, in cells with high HER2 expression, differentiation is seen at much lower concentrations of 17-AAG. Furthermore, ansamycins have been shown to induce differentiation in other cell lineages, such as colon cancer and erythroleukemia (28–30). Therefore, it is likely that other Hsp90-regulated pathways are involved.

Induction of differentiation by ansamycins is likely to be mediated via its binding to Hsp90, but other targets may also be involved. At least two other Hsp90 family members have been identified and bind ansamycins: grp94, which acts as a chaperone in the endoplasmic reticulum; and TRAP1, which is located in mitochondria (15, 31–33). The role of each of these in mediating the cellular response to ansamycins is unknown. It is possible that other targets exist as well. The normal cellular functions of the Hsp90 family are not completely understood. Hsp90 is required for the protein refolding that occurs after heat shock and other cellular stress, and it is required for the maturation of certain signaling proteins. Studies performed in Drosophila have shown that Hsp90 silences mutations, perhaps by stabilizing mutant proteins that fold inefficiently (34). Hsp90 levels increase with oncogenic transformation and are elevated in a variety of tumors including breast cancer. This increase is associated with poorly differentiated cancers (35).

Hsp90 could play several necessary roles in maintaining the unregulated growth of transformed cells. Several of the proteins responsible for transducing the growth signal require Hsp90. Furthermore, in tumors, many of these pathways are activated by gain of function mutants that may require Hsp90 for stability. Cancer cell growth may, therefore, be quite sensitive to inhibition of Hsp90-mediated folding. In these cells, differentiation may be the default pathway that occurs when the activated growth-signaling pathways are suppressed. Hsp90 could also regulate proteins that are specific controllers of mammary differentiation.

Ansamycins are potent antitumor antibiotics. Despite the spectrum of important proteins that are degraded in response to these drugs, they have antitumor activity in animals at doses that are not particularly toxic. In addition, Phase I clinical trials are ongoing, and preliminary data suggest that micromolar peak concentration can be achieved without significant toxicity. The HER2 tyrosine kinase is one of the most sensitive targets of these drugs, and breast cancer cell lines that overexpress HER2 are inhibited by 10–50 nM of 17-AAG. At these concentrations, 17-AAG causes a rapid loss of HER2 and cyclin D expression accompanied by G1 arrest, differentiation, and subsequently apoptosis. For this reason, breast cancer is an attractive target for 17-AAG, and Phase II trials are planned. The data presented here suggest that one potential mechanism of antitumor activity in breast cancer is induction of differentiation and that differentiation markers may be useful biological markers of tumor response.

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REFERENCES


Inhibition of Heat Shock Protein 90 Function by Ansamycins Causes the Morphological and Functional Differentiation of Breast Cancer Cells

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