[CANCER RESEARCH 62, 3132–3137, June 1, 2002]

Degradation of HER2 by Ansamycins Induces Growth Arrest and Apoptosis in Cells with HER2 Overexpression via a HER3, Phosphatidylinositol 3′-Kinase-AKT-dependent Pathway[1]

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ABSTRACT

Breast cancers with high expression of HER2 are associated frequently with aggressive, poor prognosis disease and resistance to chemotherapy-induced apoptosis. Geldanamycin and its less toxic analogue, 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) are ansamycin antibiotics that bind to a highly conserved pocket in the hsp 90 chaperone protein and inhibit its function. Hsp 90 is required for the refolding of proteins during environmental stress and the conformational maturation of certain signaling proteins. Among the most sensitive targets of 17-AAG are the HER kinases. Therefore, tumors that are dependent on these kinases may be especially sensitive to 17-AAG either alone or in combination with chemotherapy. In this study we demonstrate that cells that overexpress HER2 are 10–100-fold more sensitive to 17-AAG than cancer cells expressing low levels of HER2. We found that HER2 is degraded in several cell lines, but only cell lines with high levels of HER2 are sensitive to the drug. The effects of 17-AAG on growth and apoptosis are because of inhibition of signaling through HER2-HER3, phosphatidylinositol 3′-kinase. The absence of HER3 and the introduction of constitutively active p110α rendered cells with high HER2 expression more resistant to 17-AAG.

These findings suggest that 17-AAG may be useful for the treatment of breast cancer cells with high levels of HER2. However, the overexpression of HER2 alone may not be predictive of response, because the coexpression of HER3 and the activation of phosphatidylinositol 3′-kinase may play a crucial role in the response of these cells to 17-AAG and other drugs directed against HER2. These observations have important clinical implications because they may help to identify patients that are most likely to benefit from 17-AAG and may explain resistance to Herceptin as seen in many patients.

INTRODUCTION

17-AAG,[3] a modified ansamycin antibiotic, binds to a conserved pocket in the hsp 90 protein and inhibits its function (1, 2). Hsp 90 is a chaperone protein that plays an important role in the maturation of certain signaling proteins and the in refolding of proteins in cells exposed to environmental stress (3, 4). Treatment of cancer cells with 17-AAG causes a RB-dependent G_1 cell cycle block followed by differentiation and apoptosis. In cells with mutated RB, G_1 progression is unaffected by 17-AAG. Instead these cells undergo cell cycle arrest in mitosis followed by apoptosis (5). In breast cancer cells, 17-AAG enhances apoptosis induced by chemotherapy in a schedule and RB-dependent manner (6). The inhibition of hsp 90, a crucial housekeeping protein, leads to the proteasomal degradation of several important signaling proteins. However, some targets are more sensitive than others. Among the most sensitive targets are the HER kinases (7, 8). Therefore, one might expect that cancer cells that are dependent on this kinase for survival and proliferation are especially sensitive to 17-AAG.

HER2 is a member of the EGFR family that is overexpressed in about 25–30% of breast cancer and to a lesser degree in other cancers (9, 10). Survival and time to relapse are shorter in patients whose tumors overexpress HER2 (9). HER2 overexpression has been shown to enhance proliferation, survival, and possibly the metastatic potential (11). HER2 activation has been associated with activation of AKT kinase and mitogen-activated protein kinase (12, 13). This may be mediated through a PI3k pathway. Inhibition of HER kinase by a monoclonal antibody against HER2 has shown to be effective in some breast cancer patients that overexpress HER2 (14, 15).

17-AAG inhibits HER2 kinase and degrades HER2 protein at low nanomolar concentration. In this study we tested whether tumor cell lines with high HER2 expression are more sensitive to 17-AAG and elucidated the mechanism by which the HER2 degradation leads to growth arrest and apoptosis. Our data suggest that cells with overexpression of HER2 undergo growth arrest and apoptosis at concentrations 10–100-fold lower than cells without HER2 overexpression. Degradation of HER2 occurred in all of the cell lines treated with 17-AAG and was necessary but not sufficient for growth arrest and apoptosis. The effects of 17-AAG on cell growth and apoptosis were because of inhibition of signaling through a HER3, PI3k, AKT-mediated pathway. The absence of HER3 and constitutive activation of PI3k may confer resistance to 17-AAG. These findings may have important clinical implication, because they may guide the selection of patients most likely to benefit from 17-AAG. Furthermore, these results may explain why certain tumors despite high levels of HER2 are resistant to other anti-HER2 therapies.

MATERIALS AND METHODS

Cell Culture and Growth Assays. SKBr-3, BT-474, MDA-MB-361, MDA-MB-453, MDA-MB-231, T 47-D, MCF-7, and MDA-MB-468, SKOV-3, Colo-205, and MKN-7 cells were obtained from the American Type Culture Collection. Cells were maintained in a 1:1 mixture of DMEM-to-F12 medium 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.

Reagents. 17-AAG (provided by E. Sausville, National Cancer Institute) was dissolved in DMSO to a 10-mM stock solution. Final working solutions were diluted in medium to contain <0.01% of DMSO. Bis-benzimide (Hoechst 33258) and β-tubulin monoclonal antibody were purchased from Sigma Chemicals. Reagents for caspase activation assays were purchased from Oncogene Research Laboratory. Polyclonal antibodies were from Upstate Biotechnology Inc. (p85, the regulatory subunit of PI3k), from Santa Cruz (cyclin D1 and D3, and cdk4, HER2, HER3, and Raf-1), and from Cell Signaling (AKT and pAKT).

Received 10/26/01; accepted 3/28/02.

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[1] Supported by the National Cancer Institute Breast Specialized Programs of Research Excellence program Grant P50CA68425-02, the Taub Foundation, and a generous grant from the Byrne Foundation.

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[3] The abbreviations used are: 17-AAG, 17-(allylamino)-17-demethoxygeldanamycin; PI3k, phosphatidylinositol 3′-kinase; EGFR, epidermal growth factor receptor; RB, retinoblastoma; c.a., constitutively activated; DNS, data not shown; p85: regulatory domain of PI3k; p110α, catalytic domain of PI3k; CI, confidence interval; IB, immunoblotting.

Transfections. PI3k (p110α) Transfectants. The activated PI3K construct, p110α cDNA in pUSEamp was purchased from Upstate Biotechnology Inc. The expression vector contains murine PI3k p110α (activated) under the control of the cytomegalovirus promoter. The activating mutation is generated by addition of the avian src myristoylation sequence (MGSSKSKPK) at the NH_2 terminus. Two million cells were transfected with 10 μg of cDNA or empty vector and 10 μl Lipofectin reagent (Life Technologies, Inc., Rockville, MD). Experiments were performed 24 h after transfection. The MCF-7 cells transfected with full-length cDNA coding region of HER2 or empty vector were a kind gift from Dr. C. C. Benz (University of California San Francisco, San Francisco, CA; Ref. 16).

Antiproliferative Index. Cells (2×10^5) were plated onto six-well dishes and treated with the indicated drug or DMSO vehicle for 96 h. Drug and medium were exchanged every 48 h. After 96 h medium was removed, cells were washed with PBS, and harvested. Cells were counted on a Coulter Counter. Dose curves were plotted as a function of cell number *versus* concentrations. IC_{50}s were calculated at 96 h and reflected the concentration calculated at 50% inhibition of growth.

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 then treated with the respective concentrations of 17-AAG for the desired times. Apoptosis was assessed at the indicated times.

Assessment of Apoptosis. After drug treatment, adherent and supernatant cells were harvested and fixed with 3.5% paraformaldehyde for 10 min at room temperature and permeabilized with 0.2% Triton X-100 for 10 min at room temperature in 15-ml Falcon tubes. For tubulin staining, cells were incubated with a monoclonal β-tubulin antibody for 1 h at room temperature and then labeled with an Alexa-488 conjugated secondary antibody for 1 h at room temperature. Nuclei were stained with 0.5 μg/ml bis-benzimide (Hoechst 33258). Cell suspensions were then placed on glass slides and analyzed by conventional epifluorescent microscopy. Cells were evaluated for apoptotic score (apoptotic nuclei/all nuclei $\times$ 100%). Tubulin staining was used to clearly distinguish between apoptosis and mitosis. 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. SE denotes SE.

IB. 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 μM/ml of each NA_3VO_4, phenylmethylsulfonyl fluoride, and DTT, and 1 μg/ml each of leupeptin, aprotinin, and soybean trypsin inhibitor). Lysates (50 μg) were loaded onto 7–10% SDS-PAGE mini-gels. As described previously, proteins were transferred to nitrocellulose membranes and incubated with primary and secondary antibodies (5). Proteins were visualized by chemiluminescence (enhanced chemiluminescence; Amersham Corp.) on Bio-Max film (Eastman Kodak).

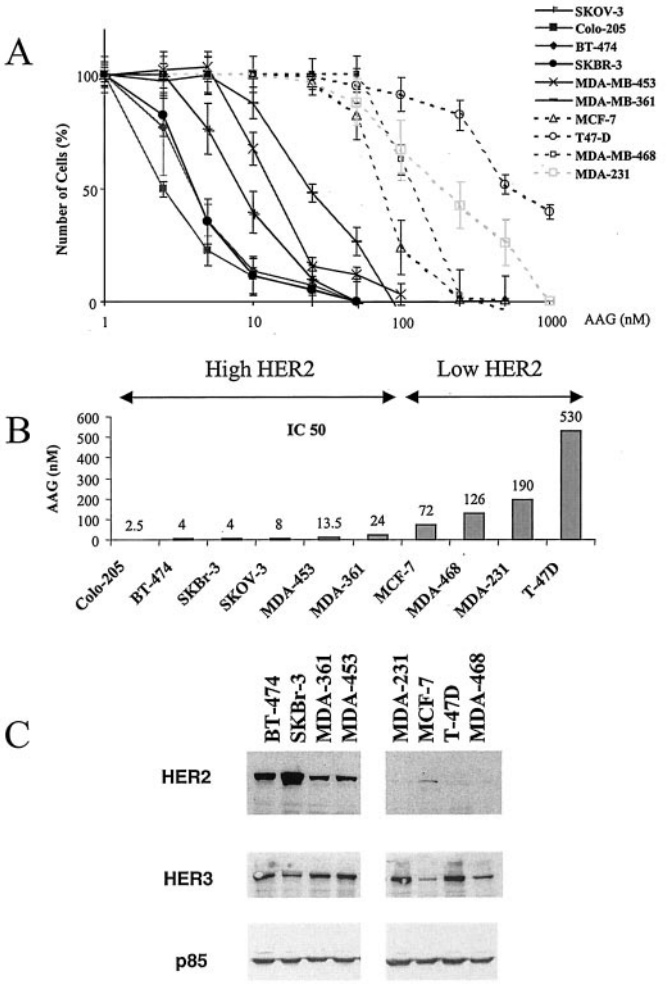

Fig. 1. Antiproliferative effects of 17-AAG and HER2 status. *A*, SKOV-3, Colo-205, BT-474, SKBr-3, MDA-MB-453, MDA-MB-361, MCF-7, T-47D, MDA-MB-468, and MDA-MB-231 cells were treated with increasing concentrations of 17-AAG for 96 h. The number of viable cells was assessed after 96 h in culture and graphed as percentage of untreated cells. *Solid lines* represent cell lines with high levels of HER2; *dotted lines* represent cell lines with low levels of HER2; *bars*, $\pm$ SE. *B*, *bar graph* of IC_{50}s: concentrations required for 50% growth inhibition at 96 h exposure. *C*, comparison of HER2, HER3, and p85 protein expression of cells at 96 h in culture determined by IB.

RESULTS

Antiproliferative Effects of 17-AAG and HER2 Status. 17-AAG causes the proteasomal degradation of several signaling proteins. One of the most sensitive targets are the HER kinase family, in particular HER2. Therefore, we evaluated whether cells that are dependent on this kinase might be more sensitive to treatment with 17-AAG. We treated a panel of cell lines with various levels of HER2 expression with increasing concentrations of 17-AAG for 96 h and evaluated the effects on cell viability. 17-AAG affected cell proliferation in all of the examined cancer cell lines, but cells with high expression of HER2 (Fig. 1*A*, *solid lines*) were more sensitive to 17-AAG than cells with low expression of HER2 (Fig. 1*A*, *dotted line*; Fig. 1*C*; DNS). The IC_{50} calculated after 96-h exposure to 17-AAG ranged from 2.5–24 nM in the cells with high HER2 expression and from 72 to 530 nM in the cells with low HER2 expression (Fig. 1*B*). Protein expression levels for HER2 were evaluated by Western blot analysis (Fig. 1*C*). The cell lines SKOV-3, Colo-205, and MKN-7 expressed HER2 levels that were comparable with SKBr-3 cells (Fig. 1*C*; DNS). Most of the breast cell lines examined also expressed elevated levels of HER3. However, elevated levels of HER3 alone did not convey sensitivity (Fig. 1*C*; DNS). Levels of p85 (the regulatory subunit of PI3k) were comparable over the range of examined cells and were not affected by 17-AAG.

Overexpression of HER2 Increases Sensitivity to 17-AAG. To additionally evaluate whether HER2 conveys sensitivity we studied the effects of 17-AAG on MCF-7 that were transfected with full-length cDNA coding region. These cells were a subclone of MCF-7 cells with significantly increased levels of HER2 expression (16). HER2 levels in the mock-transfected MCF-7 subclone were similar to wild-type MCF-7 (Fig. 2*B*). The effects of 17-AAG on MCF-7:HER2 were compared with the subclone of MCF-7 cells that were mock-transfected and to wild-type MCF-7 cells, as well as to SKBr-3, a breast cancer cell line with high endogenous levels of HER2 expression. Expression of HER2 protein levels was assessed by Western blot analysis. In the HER2-transfected cells, HER2 expression was increased by 10-fold $\pm$ 1.5. Antiproliferative effects were assessed in cells cultured with increasing concentrations of 17-AAG for 96 h (Fig. 2). The transfected MCF-7:HER2 cells were more sensitive to 17-AAG than the wild-type or the mock-transfected subclone, yet not quite as sensitive as SKBr-3 cells [IC_{50}s: SKBr-3, 4.3 nM (CI, 2.9–6.0); MCF-7:HER2, 13.3 nM (CI, 5.4–21.1), MCF-7: mock, 68 nM

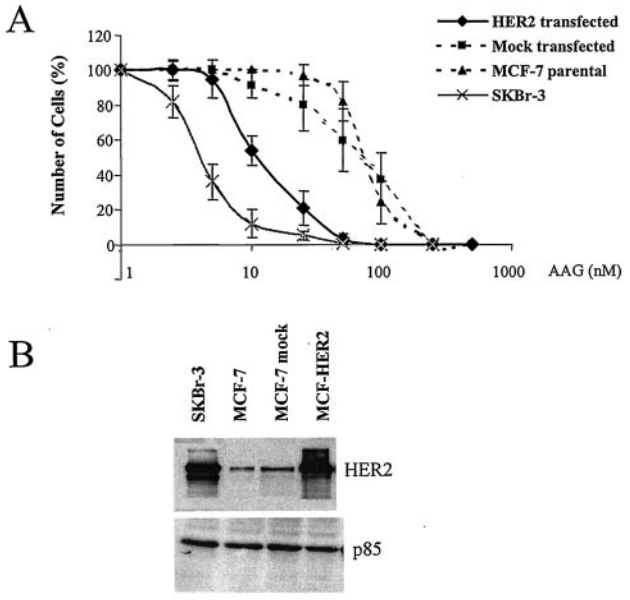

Fig. 2. Overexpression of HER2 increases sensitivity to 17-AAG. *A*, inhibitory growth effects of 17-AAG on a subclone of MCF-7 cells transfected with HER2 were compared with a mock-transfected subclone of MCF-7 cells, wild-type MCF-7 cells, and SKBr-3 cells. Cells were cultured with increasing concentrations of 17-AAG, and the number of viable cells was evaluated at 96 h and graphed as percentage of untreated cells as described in "Materials and Methods;" *bars*, ± SE. *B*, comparison of HER2 and p85 (PI3k regulatory subunit) protein expression levels of untreated cells determined by IB.

(CI, 30.1–105.9); and MCF-7 wild-type, 72 nM (CI, 47.8–95.4)]. SKBr-3 cells have only moderate levels of HER3.

Degradation of HER2 Is Necessary but not Sufficient to Induce Growth Arrest and Apoptosis. We have shown above that cells that overexpress HER2 are more sensitive to 17-AAG-induced growth arrest and apoptosis, and that overexpression of HER2 by transfection into cells with low endogenous HER2 expression renders these cells more sensitive to 17-AAG. These findings could be explained by two mechanisms; either drug uptake is different in these cells, or the cellular signaling pathways are regulated and affected differently. Therefore, we treated cells with either high levels (SKBr-3 and BT-474) or low levels of HER2 (MCF-7 and MDA-231) with concentrations of 17-AAG sufficient to cause growth arrest and evaluated the drug effects on apoptosis. We have shown above that the IC_{50} for SKBr-3 and BT-474 was <5 nM, whereas the IC_{50} for MCF-7 was 72 nM and 190 nM for MDA-231. Apoptotic nuclei were scored as described in "Materials and Methods." At 48 h, 50 nM 17-AAG caused significant apoptosis in SKBr-3 and BT-474 cells (24% ± 5 and 18% ± 2) but not in MCF-7 and MDA-231 cells (0.5% ± 0.25 and 1% ± 0.5; Fig. 3*A*). We then evaluated the effects of 50 nM 17-AAG on hsp 90 client proteins such as HER2 and Raf-1. An ansamycin-induced decrease in Raf-1 expression has been well described by several investigators (17–21). Analysis of protein expression of these cells treated for 0, 4, 12, and 24 h with 17-AAG showed that Raf-1 expression was decreased by >90% by 24 h in all of the examined cell lines irrespective of their levels of HER2 expression. This suggests that the increased sensitivity in the high HER2-expressing cell lines is unlikely because of a difference in cellular drug uptake. Furthermore, at 24 h a >90% decrease in expression of HER2, HER3, and HER4 was found in all of the examined cell lines that did not correlate with the antiproliferative and proapoptotic effects of the drug (Fig. 3*B*; DNS). Loss of protein expression occurred with similar kinetics in all four of the examined cell lines. In contrast, expression of cyclin D1 was significantly decreased by 24 h only in the cells with high expression of HER2. We have found that whereas cyclin D levels were not decreased in parental MCF-7 cells, in the MCF-7 cells that were transfected with HER2, cyclin D was decreased (Fig. 3*C*). As described above, the MCF:HER2 cells are more sensitive to 17-AAG than the parental cells, however, they are not as sensitive as SKBr-3 cells. Similarly, the 17-AAG-mediated effects on cyclin D levels were not as pronounced and occurred later (Fig. 3*C*). We have reported previously that cyclin D1 is not a direct target of 17-AAG but is regulated by AKT (22). We and others have shown that the effects on AKT are most likely mediated through heterodimerization of HER2 with HER3 and subsequent signaling through a PI3k-mediated pathway (13, 23, 24). Compared with other HER-family members, HER3 contains six docking sites for p85 (25, 26). Immunoprecipitation with HER1, HER3, and HER2 showed an association of p85 with HER3 but not with HER2 or HER1 (DNS). Therefore, we speculated that the

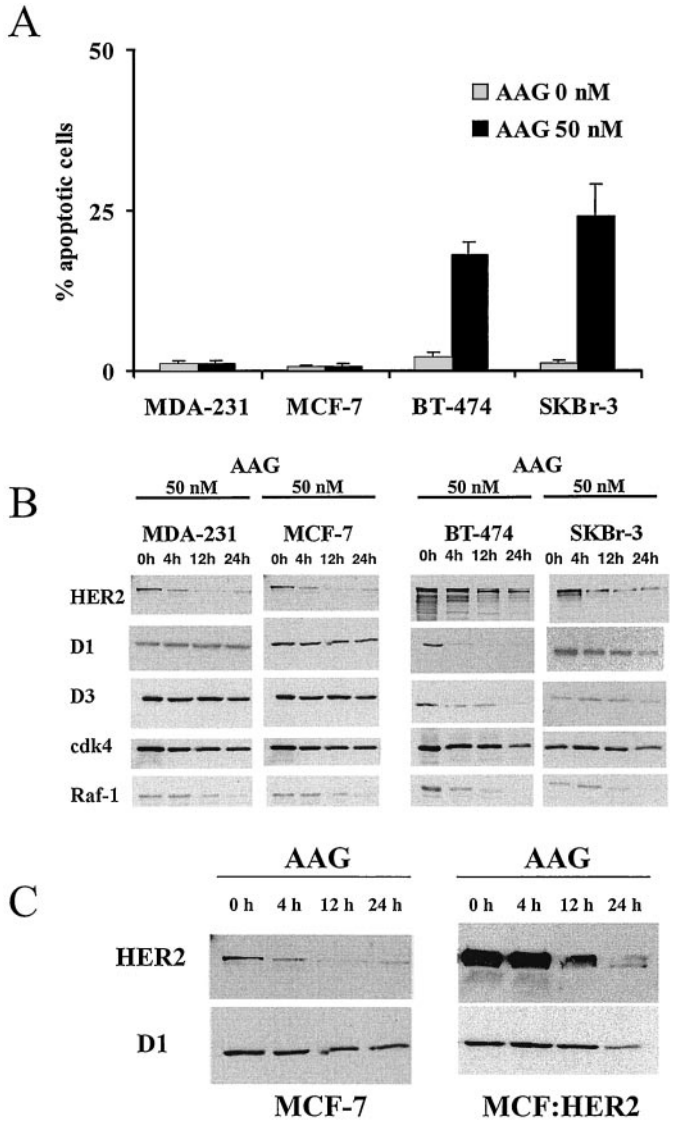

Fig. 3. Degradation of HER2 is necessary but not sufficient to induce apoptosis. *A*, effects of 17-AAG on cells at concentrations sufficient to cause HER2 degradation. Two breast cancer cell lines with low HER2 (MCF-7 and MDA-231) and two cell lines with high HER2 expression (BT-474 and SKBr-3) were treated with 0 or 50 nM 17-AAG for 48 h. Nuclei were assessed for apoptotic score by quantitative fluorescence microscopy at 48 h using bis-benzimide and antitubulin staining as described in "Materials and Methods;" *bars*, ± SE. *B*, time course of effects of 17-AAG on protein expression determined by IB. Cells were treated with 50 nM 17-AAG for 0, 4, 12, and 24 h. *C*, time course of effects of 17-AAG on protein expression determined by IB in MCF-7 cells, parental cells, and MCF-7 cells transfected with HER2 (MCF:HER2). Cell were treated with 50 nM 17-AAG for 0, 4, 12, and 24 h.

17-AAG-induced growth arrest and apoptosis in these cells is because of a loss of HER2 and HER3 activity, and protein expression with subsequent loss of association and inhibition of PI3k and AKT activity. We have shown here (Figs. 1 and 2) and elsewhere (23) that at 50 nM 17-AAG, PI3k (the regulatory form p85 and the activating form p110), and AKT activity are not directly affected by the drug.

To support this observation we tested whether the cellular effects of 17-AAG were abrogated in SKBr3–3 cells with c.a. p110α.

c.a. p110α Decreases the Apoptosis and Growth Arrest Induced by 17-AAG in Cells with High HER2 Levels. SKBr-3 cells were transfected with an expression vector containing an activated murine PI3k p110α. The activating mutation was generated by addition of the avian src myristoylation sequence (MGSSKSKPK) at the NH_2 terminus as described in "Materials and Methods." After induction of p110α we evaluated the effects of 17-AAG on growth in these transfected cells, as compared with mock-transfected cells and wild-type SKBr-3. The antitumor activity was assessed after cells had been exposed to increasing concentrations of 17-AAG for 96 h. We found that compared with the mock-transfected cells, the p110α-transfected SKBr-3 cells became less sensitive. The minimal concentrations required for complete growth arrest ($\geq$95% suppression of cell growth) were as follows: mock transfected: $\geq$25 nM; p110α-transfected: $\geq$100 nM; and wild-type SKBr-3: $\geq$25 nM. These findings suggest that activation of p110α decreases the antiproliferative effects of 17-AAG.

We subsequently evaluated whether c.a. p110α affected only the 17-AAG-induced inhibition of growth or whether it also inhibited 17-AAG-induced apoptosis. We have shown previously that significant induction of apoptosis by 17-AAG was not observed before 48 h of 17-AAG exposure (5). Induction of apoptosis in SKBr-3 cells transfected with c.a. p110α as described above was compared with mock-transfected SKBr-3 cells. Exposure of cells for 48 h with 50, 100, and 500 nM 17-AAG caused significantly fewer apoptotic nuclei in SKBr-3:p110α (50 nM: 17 *versus* 31%, $P = 0.003$; 100 nM: 26 *versus* 40%, $P = 0.02$; 500 nM: 31 *versus* 42%, $P = 0.016$; Fig. 4*B*). Whereas the differences were statistically significant at all concentrations of 17-AAG, the effects were more pronounced at lower concentrations.

Absence of HER3 Conveys Resistance to 17-AAG. To additionally demonstrate the importance of HER2-HER3 heterodimerization and its subsequent signaling through a PI3k, AKT kinase-mediated pathway, we studied a cell line that has high levels of HER2 expression but does not express HER3. We compared the effects of 50 nM 17-AAG on the growth of MKN-7, a gastric cell line with very high expression of HER2 but low-absent HER3 expression (24). We compared MKN-7 cells to BT-474 and SKBr-3, two breast cancer cell lines, the MCF-HER2 transfected cell line, and to Colo-205, a colon cell line with high levels of HER2. As seen in Fig. 4*C*, 96-h exposure to 17-AAG resulted in >95% fractional inhibition of cell growth in all of the examined cell lines except in the MKN-7 cells. These cells were only minimally affected by 50 nM 17-AAG suggesting a crucial role of HER3 for the antiproliferative and apoptotic effects of 17-AAG on cancer cells with high expression of HER2.

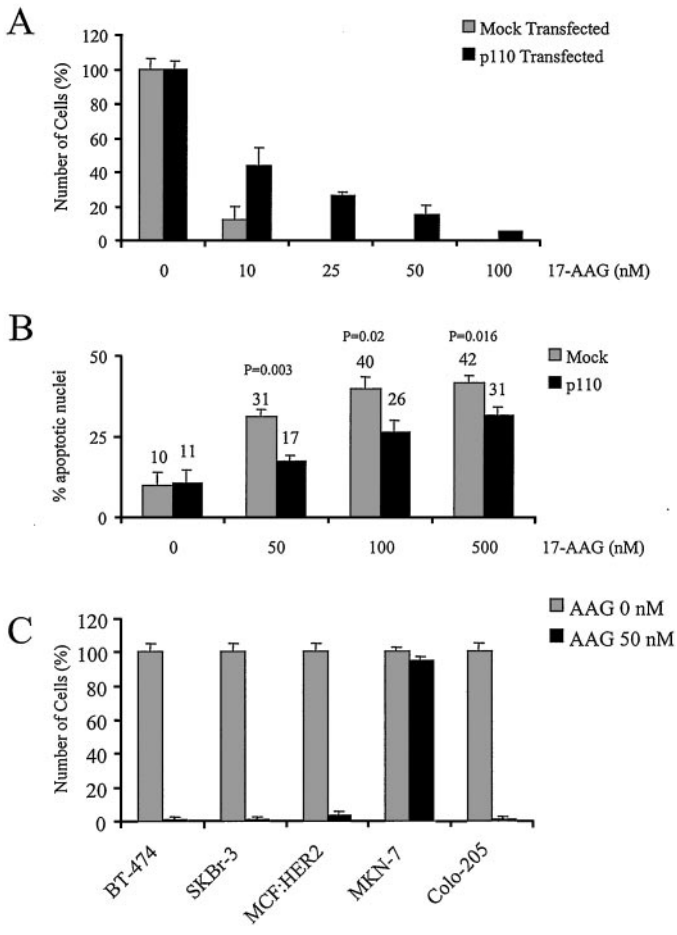

Fig. 4. c.a. p110α decreases growth arrest and apoptosis induced by 17-AAG in cells with high HER2 levels. *A*, antiproliferative effects of 17-AAG on p110α transfected cells. Mock-transfected and p110α-transfected SKBr-3 cells were cultured with 0, 10, 25 50, and 100 nM 17-AAG for 96 h. The number of viable cells was assessed at 96 h and graphed as percentage of untreated cells. *B*, 17-AAG induced effects on apoptosis in p110α-transfected SKBr-3 cells. Mock-transfected and p110α-transfected SKBr-3 cells were cultured with 0, 50, 100, and 500 nM 17-AAG for 48 h. Cells were harvested and assessed for apoptotic score as described in "Materials and Methods." *C*, effects of 17-AAG in the absence of HER3. The growth inhibitory effects of 17-AAG on MKN-7 cells (gastric: high HER2, absent HER3) were compared with cells that overexpress HER2 and HER3 (SKBr-3, BT-474, MCF-7HER2: breast, colo-205: colon). Cells were cultured with 50 nM 17-AAG for 96 h; the number of viable cells was assessed at 96 h and graphed as percentage of untreated cells; *bars*, $\pm$ SE.

DISCUSSION

This study demonstrates that in cells with high expression of HER2, treatment with 17-AAG lead to growth arrest and apoptosis at nanomolar concentration. Treatment of these cells with concentrations of <50 nM caused degradation of HER2 associated with rapid loss of cyclin D1 expression. In addition, 17-AAG treatment resulted in inactivation of AKT and activation of the caspases 3 and 9 (6, 23). In contrast, in cells with low or absent expression of HER2, growth arrest required concentrations that were 20–200-fold higher and apoptosis was much less prominent and occurred later. In these cell lines, exposure to drug at concentrations that were sufficient to degrade HER2 did not result in growth arrest, apoptosis, despite concomitant degradation of other client proteins of hsp 90, such as Raf-1, hsp 70, and other HER kinase family members (HER3 and EGFR). Our results suggest that PI3k, and AKT activity and cyclin D expression are not direct targets of 17-AAG, and therefore must be regulated by upstream targets. Introduction of HER2 into cells with low expression of HER2 increased the sensitivity, whereas absence of HER3 expression rendered cells more resistant to the drug. Constitutive activation of p110α decreased sensitivity to 17-AAG-induced growth arrest and apoptosis, and in part abrogated sensitization to cytotoxic agents. Introduction of p110α did not alter chemotherapy-induced apoptosis.

Ansamycins are novel anticancer agents that inhibit hsp 90 by occupying its NH_2-terminal ATP-binding site (1, 2). Hsp 90 is involved in both maturation of a number of key signaling proteins and the refolding of proteins in cells exposed to stress (3, 4, 7, 8, 27, 28). The expression of hsp 90 is induced when cells are exposed to heat.

Inhibition of hsp 90 by ansamycins has been shown to degrade several key signaling proteins (4, 7, 8, 27, 28). However, this study shows that whereas several proteins are affected by the inhibition of hsp 90, only the effects on a few proteins appear to be responsible for growth arrest or apoptosis, and probably only in tumors that are dependent on these proteins for cell survival. These findings have two important clinical implications. It may guide us to identify tumors that are more sensitive to 17-AAG. Furthermore, changes in client proteins of hsp 90 that have been studied in the currently ongoing clinical trials with 17-AAG may have to be reevaluated. The effects on some of the hsp 90 client proteins may represent sufficient drug levels but not necessarily correlate with antitumor effects.

HER2 is expressed in all of the breast cancer cells. We found that at concentrations of 17-AAG that are sufficient to degrade HER2, only tumors with high expression of HER2 are growth arrested and undergo apoptosis. In cells with low expression of HER2, higher concentrations were required and apoptosis occurred later, after 72–96 h of drug exposure. Furthermore, we found that 50 nM of 17-AAG did not cause growth arrest in MKN-7 cells. These cells have high expression of HER2 but lack HER3 expression. MKN-7 cells have been reported to be resistant to Herceptin (24). These findings may suggest a role of HER3 in HER2 signaling; however, this remains speculative, and more extensive experiments are needed. It is of interest that EGFR is overexpressed in MKN-7 cells, suggesting that EGFR alone appears to be insufficient to activate HER2. On the other hand, MCF-7 cells that are fairly resistant become more sensitive to 17-AAG when HER2 is overexpressed by transfection, however, not to the same degree as other breast cancer cells with high HER2 expression. The findings suggest that at least in the examined clone, the introduction of HER2 into fully transformed MCF-7 cells appears to change the phenotype and signaling pathway. Our data suggest that in the parental MCF-7, degradation of HER2 was not sufficient to arrest growth and cause apoptosis. In contrast, in the transfected MCF-7 cells, the decrease in HER2 and cyclin D expression was associated with growth arrest suggesting that these cells have become dependent on HER2 for proliferation. However, additional experiments are needed to elucidate the basis of this phenomenon.

HER2 signaling through HER2-HER3 with activation of PI3k and AKT has been suggested by other investigators (13, 23, 29). Activation of AKT has been implicated to promote tumor cell proliferation and to inhibit apoptosis. A role of HER3 in the activation of AKT has been reported by others and is not limited to breast cancer but may be important in nonmelanomatous skin cancer (30) and gastric cancer (13). On the other hand it has long been established that transformation induced by HER3 and HER4 requires HER2 (31).

This study suggests that the effects of 17-AAG on cells with high levels of HER2 may be explained by its effects on AKT activity. We have shown previously that treatment of cells with 17-AAG results in rapid loss of p-AKT in cells with high HER2 expression (23). 17-AAG does not directly affect the regulatory p85 or the catalytic p110 subunits of PI3k. Furthermore, we have shown previously that 17-AAG does not directly affect *in vitro* AKT kinase. HER3 has at least six docking and activation sites for PI3k, whereas thus far HER2 is not believed to be associated with PI3k (25, 26). Therefore, we propose that the 17-AAG-induced cellular effects are because of loss of HER2 and HER3 expression with subsequent inactivation of PI3k and AKT in cells that are dependent on this pathway for cell proliferation and inhibition of apoptosis. This is supported by the findings that c.a. p110α renders the sensitive SKBr-3 cells more resistant to 17-AAG. However, p110α transfection only partially abrogated the effects of 17-AAG. Our experiments were performed with transient overexpression of p110α. In these experiments, AKT was activated up to 96 h after transfection. Transfection efficiency was around 45–60% at this time point. This should be considered as one of the reasons for the partial reversal of 17-AAG effects. It has been reported that stable expression of c.a. p110α alters cellular morphology or may cause differentiation but does not result in maintained activation of AKT (32, 33). Preliminary data from our experiments suggested that stable transfection of SKBr-3 with c.a. p110α altered cellular morphology and resulted in cell death. Therefore, the reported experiments were performed with transient transfection.

While these findings are intriguing, the experiments were performed in select cell types, and these results have not been evaluated in the context of p53 and RB mutations or loss of PTEN.

We propose that the effects on growth and apoptosis induced by 17-AAG in cells with high levels of HER2 is because of its inhibition of HER2 and HER3 with subsequent inhibition of PI3k, AKT, and cyclin D. These findings may have important clinical implications. The results of this study suggest that patients, whose tumors overexpress high HER2, are more likely to be sensitive to 17-AAG. Furthermore tumor samples of patients treated with 17-AAG or other anti-HER2 therapy, in particular Herceptin, should be evaluated for HER3 expression and activation of PI3k. Whereas most of this work has been done in breast cancer cells, 17-AAG may be useful for the treatment of other solid tumors dependent on HER2 for cell survival.

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Cancer Res 2002;62:3132-3137.