Inhibition of Signal Transduction by the Hsp90 Inhibitor 17-Allylamino-17-demethoxygeldanamycin Results in Cytostasis and Apoptosis[1]

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ABSTRACT

17-Allylamino-17-demethoxygeldanamycin (17AAG) is a first-in-class heat shock protein 90 (Hsp90) molecular chaperone inhibitor to enter clinical trials. The downstream molecular and cellular consequences of Hsp90 inhibition are not well defined. 17AAG has shown activity against human colon cancer in cell culture and xenograft models. In this study, we demonstrated that in addition to depleting c-Raf-1 and inhibiting ERK-1/2 phosphorylation in human colon adenocarcinoma cells, 17AAG also depleted N-ras, Ki-ras, and c-Akt and inhibited phosphorylation of c-Akt. A consequence of these events was the induction of cell line-dependent cytostasis and apoptosis, although the latter did not result from dephosphorylation of proapoptotic Bad. One cell line, KM12, did not exhibit apoptosis and in contrast to the other cell lines overexpressed Bag-1, but did not express Bax. Taken together with other determinants of 17AAG sensitivity, these results should contribute to a more complete understanding of the molecular pharmacology of 17AAG, which in turn should aid the future rational clinical development and use of the drug in colon and other tumor types.

INTRODUCTION

One promising therapeutic strategy for treating cancer is to specifically target signal transduction pathways that have a key role in oncogenic transformation and malignant progression (1, 2). The benzoquinone ansamycin antibiotics, such as geldanamycin and herbimycin A, have been identified as agents that revert transformation by v-Src and exhibit potent antitumor activity (3–6). Subsequent studies have revealed that inhibition of c-Src catalytic activity, the expected mechanism of action, was not responsible for antitumor activity (6, 7). Affinity binding experiments identified the chaperone Hsp90[3] as one of the primary targets of geldanamycin (6). Hsp90 has an important role in maintaining the correct conformation and stability of a number of client proteins, but it is less promiscuous than other chaperones in that the number of its client proteins is limited (8). The interaction of geldanamycin with Hsp90 results in competition for ATP binding to Hsp90 and inhibition of its chaperone function, a consequence being the ubiquitination and degradation of client proteins by the proteasome pathway (6). The antitumor activity of geldanamycin has been attributed to destabilization of key client proteins, including receptor and nonreceptor kinases (Erb-B2, epidermal growth factor receptor, and Src family kinases), serine/threonine kinases (c-Raf-1 and Cdk4), steroid hormone receptors (androgen and estrogen), and cell cycle and apoptosis regulators such as mutant p53 (5, 6). Unfortunately, geldanamycin has limited clinical potential because of its excessive liver toxicity (5). However, subsequent derivatization of geldanamycin has yielded 17AAG, an analogue with reduced liver toxicity that retains the potent antitumor activity of the parent compound (3, 9, 10). This derivative has very recently entered Phase I clinical trial as the first-in-class Hsp90 inhibitor at our center and in the US, under the auspices of the United States NCI and the United Kingdom CRC.

Mutation and activation of Ki-ras occurs in ~30% of all colon cancers, and certain mutations are associated with a poorer outcome (11). In addition, constitutive activation of ERK-1/2 is common in colon cancers, and inhibitors of the Ras/Raf/MEK/ERK signaling pathway effectively inhibit the growth of colon cancer cells *in vitro* and *in vivo* (12, 13). Given that 17AAG depletes c-Raf-1 and inhibits ERK-1/2 phosphorylation, we hypothesized that 17AAG could be an effective anticancer agent against colon cancer, among other tumor types. Indeed, we have recently demonstrated that 17AAG inhibits the growth of human ovarian and human colon carcinomas *in vitro* and delayed the growth of two colon cancer xenograft lines *in vivo* (3).

Inhibition of signal transduction by agents such as 17AAG could potentially result in cytostasis or cell death, and the balance of these biological fates could influence patient treatment strategy and clinical outcome. For example, a cytostatic response would require a chronic administration schedule, as is used for various signal transduction inhibitors (*e.g.,* Ref. 13), whereas a cell-killing effect might allow a more intermittent dosing regimen. Moreover, understanding the molecular basis of these responses to 17AAG might provide pharmacodynamic end points and the identification of factors that could lead to a selection of patients most likely to be responsive to the drug. Although we have previously shown that 17AAG is active against colon cancer *in vitro* and *in vivo*, the signaling pathways affected by 17AAG in colon cancer are not well understood and the ability of this agent to induce apoptosis has not been determined in any tumor model. This present study is the first to show that in human colon adenocarcinoma cells, 17AAG depletes c-Raf-1 and inhibits ERK-1/2 phosphorylation, as has been described for other tumor types (3, 6, 10). Moreover, we also demonstrate that treatment of human colon adenocarcinoma cells with 17AAG results in depletion of N-ras, Ki-ras, and c-Akt together with inhibition of c-Akt phosphorylation. Furthermore, we show that 17AAG treatment results in both cytostasis and apoptosis to an extent depending on the particular colon cancer cell line, and we identify factors that may influence and regulate these responses.

MATERIALS AND METHODS

Tissue Culture. Human colon adenocarcinoma cell lines (HCT15, HCT116, HT29, and KM12) were cultured in DMEM (Life Technologies, Inc., Paisley, United Kingdom) supplemented with 10% FCS (PAA Laboratories, Kingston, United Kingdom), 1× nonessential amino acids (Life Technologies), and 2 mM L-glutamine in a 5% CO_2 atmosphere. Cells in mid-log phase of growth were exposed to 17AAG (kindly supplied via Dr. E. Sausville, NCI, Bethesda, MD) for 24 h. The cells were then washed and supplemented with fresh drug-free medium and harvested for molecular analysis either immediately or at 48 and 72 h. The viability of adherent cells was measured at 72 h by trypan blue exclusion.

Analysis of Protein. Cells were resuspended in lysis buffer [150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.2% (w/v) SDS, 0.2% (v/v) NP40, 1% (v/v) glycerol, 1 mM EDTA, 0.5 mM sodium orthovanadate, 10 mM PP$_i$, 100 mM NaF, and 1× protease inhibitors (Complete Mini; Roche Diagnostics, Indianapolis, IN)] and an equal volume of 80 mM Tris-HCl containing 6 mM MgCl$_2$. An aliquot was removed for protein estimation by Lowry assay (Bio-Rad, Hercules, CA). Equal amounts of protein and Rainbow molecular weight markers (Amersham Pharmacia Biotech, Amersham, United Kingdom) were separated by electrophoresis through polyacrylamide gels and electrotransferred to Hybond-C nitrocellulose (Amersham Pharmacia Biotech). Immunoblots were blocked with 5% nonfat milk in TBST1 [10 mM Tris-HCl (pH 7.6), 142 mM NaCl, 0.1% Tween-20] and then incubated with 0.4 μg/ml each of anti-c-Raf-1 rabbit polyclonal antibody, anti-Bag-1 rabbit polyclonal antibody (Santa Cruz Biotechnology Biotech, Santa Cruz Biotechnology, CA), anti-N-ras mouse monoclonal antibody, and anti-Ki-ras mouse monoclonal antibody (Oncogene Research, Cambridge, MA); 1 μg/ml each of anti-ERK-1/2 rabbit polyclonal antibody, anti-phospho-ERK-1/2 rabbit polyclonal antibody, anti-c-Akt rabbit polyclonal antibody, and anti-phospho-c-Akt Ser-473 mouse monoclonal antibody (New England Biolabs, Beverly, MA); 0.5 μg/ml anti-phospho-ERK-1/2 mouse monoclonal antibody (Sigma-Aldrich, St. Louis, MO); 20 μg/ml anti-GAPDH mouse monoclonal antibody (Chemicon, Temecula, CA); 0.5 μg/ml anti-Bad mouse monoclonal antibody (Transduction Labs, Lexington, KY); 2 μg/ml anti-PARP mouse monoclonal antibody (Clontech, Palo Alto, CA); 1 μg/ml anti-caspase 3 rabbit monoclonal antibody; a 1:1000 dilution of anti-Bax rabbit polyclonal antibody (BD PharMingen, San Diego, CA); and a 1:70 dilution of anti-Bcl-2 mouse monoclonal antibody (Dako, Ely, United Kingdom) diluted in 5% milk powder in TBST1. Specific antigen-antibody interactions were detected with horseradish peroxidase-conjugated goat antimouse or antirabbit IgG and enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech).

Immunoprecipitation. Cell lysates were precleared by addition of 10 μl of 4 mg/ml protein G-Sepharose beads (Sigma-Aldrich) for 1 h at 4°C. Phospho-Bad was immunoprecipitated from 7 mg of protein in lysis buffer with 1:200 dilution of a rabbit polyclonal antibody specific to Ser-136 phospho-Bad or a 1:200 dilution of a rabbit polyclonal antibody specific to Ser-112 phospho-Bad (New England Biolabs, Beverly, MA). The antibody-antigen complex was recovered by the addition of 20 μl of protein G-Sepharose to the cell lysate and incubation for 1 h at 4°C. The beads were washed twice with TBST2 [10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.05% Tween 20], then once with TBST2 and once with 0.05 M Tris-HCl (pH 6.8). Immunoprecipitated phospho-Bad was detected by Western blotting with an antibody that recognizes phosphorylated and nonphosphorylated Bad as described earlier.

Morphological and Cell Cycle Analysis. Cells were grown and treated in 24-well plates on 13-mm glass coverslips and fixed for 1 h at room temperature in 4% *p*-formaldehyde in PBS. Fixed cells were permeabilized with 0.5% Triton X-100 for 10 min and incubated with 10 μg/ml RNase A and 1 μg/ml propidium iodide for 15 min at 37°C; the coverslips were then mounted in VectaSheild (Vectorlabs, Peterborough, United Kingdom). Images were collected using a Leica SP confocal microscope (Leica, Milton Keynes, United Kingdom).

DNA histograms were generated by fluorescence-activated cell sorting analysis. Cells (1 × 10^6) were washed in PBS and fixed in 70% ethanol for 30 min at 4°C. The fixed cells were washed once with PBS, resuspended in PBS containing 100 μg/ml RNase A and 40 μg/ml propidium iodide, and incubated for 30 min at 37°C. All samples were analyzed on an Ortho Cytoron Absolute flow cytometer (Ortho Clinical Diagnostics, Amersham, United Kingdom). Data were analyzed and histograms plotted using winMDI version 2.5 flow cytometry application software (Scripps Institute, La Jolla, CA).

RESULTS

Four human colon adenocarcinoma cell lines, HT29, HCT116, KM12, and HCT15, were chosen for this study. These cell lines were selected because they are all members of the NCI 60-cell line panel and their mRNA expression profile in response to 17AAG had been documented previously (14). We previously have shown that the antitumor activity of 17AAG may be influenced by the expression of the quinone reductase DT-diaphorase and that cells carrying a poly-

morphism that disables DT-diaphorase are particularly insensitive to 17AAG (3). HT29, HCT116, KM12, and HCT15 do not have the polymorphism and express relatively similar, high levels of DT-diaphorase (14, 15). Therefore, DT-diaphorase is unlikely to contribute in a major way to any difference seen between the four colon cancer cell lines.

The cell lines were treated with a range of 17AAG concentrations for 24 h, followed by incubation in the absence of drug. This allowed analysis of molecular response and recovery to the drug. Fig. 1 shows dose-response data for the four colon cancer cell lines. The IC$_{50}$ values for adherent cell viability at 72 h, calculated from three independent experiments, were 0.2 μM (HT29), 0.8 μM (HCT116), 0.9 μM (KM12), and 46 μM (HCT15). There was evidence that geldanamycin and 17AAG are substrates for efflux by P-glycoprotein. This may potentially be a contributing factor to the high IC$_{50}$ value observed for HCT15 cells because these have a MDR phenotype (3).

Depletion of the client protein c-Raf-1 as a consequence of Hsp90 inhibition by 17AAG has been described in SKBR3 human breast cancer cells and HT29 and BE human colon cancer cells (3, 10). Therefore, we initially validated the molecular effects of 17AAG by measuring c-Raf-1 protein following 24 h of treatment with 17AAG and compared the concentration of 17AAG required to deplete c-Raf-1 to the IC$_{50}$ values determined from Fig. 1. Fig. 2*A* shows typical results obtained with HT29 cells. These molecular effects, like the others reported herein, were very reproducible within a given cell line, but replicated differences were seen between different cell lines. The minimum 17AAG concentration required to deplete c-Raf-1 by >95% was 0.5 μM for HT29, 1 μM for HCT116 and KM12, and 50 μM for HCT15 cells. Thus, the concentration required to deplete c-Raf-1 at 24 h was ~1–2 times the IC$_{50}$ for viability at 72 h. For all subsequent experiments, we used the pharmacologically relevant isoeffective doses of 0.5 μM for HT29, 1 μM for HCT116 and KM12, and 50 μM for HCT15 cells.

Interestingly, the time course of recovery from c-Raf-1 depletion following removal of 17AAG at 24 h varied among cell lines. c-Raf-1 depletion was maintained for at least 2 days following a 24-h treatment of HT29 cells, whereas HCT116 cells recovered normal c-Raf-1 protein levels within 2 days and the other two cell lines, HCT15 and KM12, showed intermediate recovery times with partial recovery at 48 h and almost complete recovery at 72 h (Fig. 2*B*).

The effect of 17AAG on other key signaling molecules was also determined. Fig. 2*C* illustrates the response of HCT116 at 24 and 48 h as an example; the data are summarized for all cell lines in Table 1. Although very reproducible for a given protein within each cell line, the kinetics of molecular response and recovery were different between cell lines and also between individual proteins. Expression of a
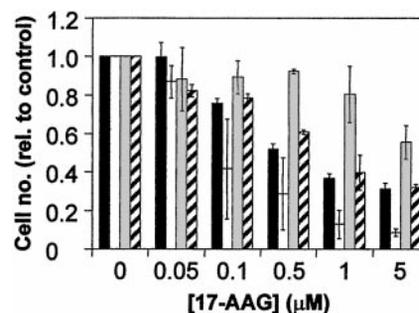

Fig. 1. HCT116 (■), HCT15 (▦), HT29 (□), and KM12 (▨) cells were treated with a range of 17AAG concentrations for 24 h and then cultured in the absence of 17AAG for an additional 48 h. The number of viable adherent cells relative to untreated controls was plotted against 17AAG concentration. Results are mean values ± SE (*bars*) of three independent experiments.
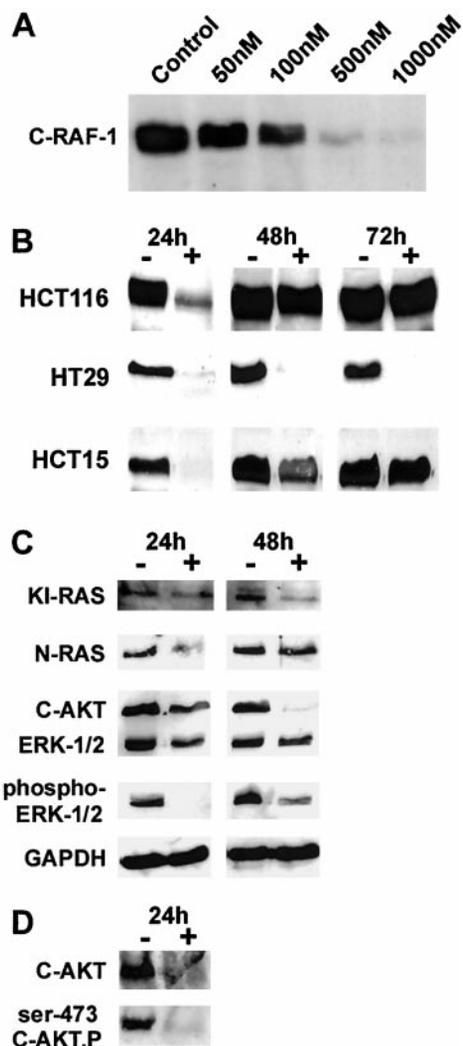
Fig. 2. Molecular effects of 17AAG on human colon cancer cells. *A*, c-Raf-1 protein expression in HT29 cells treated for 24 h with 17AAG. *B*, c-Raf-1 protein expression in HT29, HCT116, and HCT15 cells at 24, 48, and 72 h after treatment with 0.5, 1, and 50 μM 17AAG, respectively. KM12 cells show results very similar to those for HCT15 cells. *C*, protein expression of Ki-ras, N-ras, c-Akt, and ERK-1/2 and phosphorylation of ERK-1/2 at 24 and 48 h after treatment of HCT116 cells with 1 μM 17AAG. GAPDH is shown as a control. *D*, expression of c-Akt and phosphorylation of c-Akt at Ser-473 after 24-h treatment of KM12 cells with 1 μM 17AAG. −, control untreated cells; +, 17AAG-treated cells. All blots were duplicated in two independent experiments.

control protein, GAPDH, was unaffected in all cells. Ki-ras expression was decreased in all of the cell lines, and this was maintained for 3 days. A similar pattern of N-ras depletion was detected in all cell lines, except that HCT116 cells recovered normal N-ras levels at 72 h. We could not extend this observation to Ha-ras because we were unable to obtain a specific Ha-ras antibody of sufficient quality for protein analysis (data not shown). ERK-1/2 protein expression was decreased in HT29 and KM12 cells, but to a lesser extent and at a slower rate than c-Raf-1 and Ras. ERK-1/2 was depleted only marginally by 17AAG treatment of HCT116 cells and was not affected in HCT15 cells. Phosphorylation of ERK-1/2 rapidly decreased to undetectable levels in all of the cell lines tested and recovered at 48 h in HCT116 and at 72 h in HCT15 cells. c-Akt, which functions downstream in the PI3 kinase pathway, was also extensively depleted in all of the cell lines, but recovered after 72 h in HCT116, KM12, and HCT15 cells. Constitutive phosphorylation of c-Akt on Thr-308 could not be detected by Western blotting or immunoprecipitation (data not shown). Constitutive phosphorylation of Ser-473 could be detected reliably only in KM12 cells, and this was inhibited by 17AAG

treatment (Fig. 2*D*). Overall, the results clearly show that 17AAG affects several important players in oncogenic signal transduction, resulting in the inhibition of both the Ras→ERK pathway and the PI3 kinase pathway in human colorectal cancer cells.

Treatment of all of the cell lines in the study with 17AAG resulted in a concentration-dependent inhibition of adherent cell proliferation and a concomitant concentration-dependent increase in floating cells in all of the cell lines (Fig. 3). HT29 and HCT116 cells exhibited an especially large increase in the number of floating cells. Interestingly, KM12 cells showed the lowest increase in floating cells, despite the fact that they had an IC_{50} for viability at 72 h similar to that of HCT116 cells and that c-Raf-1 was completely depleted by 1 μM 17AAG in both cell lines. Fig. 3*B* illustrates the time course for the appearance of floating cells following treatment with the isoeffective concentrations of 17AAG (given above). Again, KM12 cells had the lowest number of floating cells following 17AAG treatment. Treatment of HCT116 cells induced a large increase in floating cells at 48 h and a further increase at 72 h. HCT15 and HT29 cells showed only a modest increase in floating cells at 48 h and a much larger increase at 72 h.

Morphological analysis of the small number of floating cells always present in all untreated cells revealed a mixture consisting of mitotic and apoptotic cells (data not shown). In contrast, the floating cells from the 17AAG-treated cultures had a predominantly apoptotic appearance. As examples, Fig. 4*A* shows the typical morphology of control and treated adherent HT29 and HCT116 cells together with floating HT29 and HCT116 cells following 17AAG treatment. The 17AAG-treated adherent HT29, KM12, and HCT15 cells also had a large number of mitotic figures, consistent with an increase in cells in G_2-M phase of the cell cycle (Fig. 4*A* shows an example of HT29 cells). This was also confirmed by flow cytometric cell cycle analysis, which revealed an accumulation of cells in G_2-M phase at 24 and 48 h following treatment of HT29, HCT15, and KM12 cells (Fig. 4, *B–D*), although this effect was no longer detected at 72 h (data not shown). HCT116 cells were an exception and showed little evidence for an increase in mitotic figures or an accumulation of adherent cells in either G_2-M or G_1-S following 17AAG treatment (Fig. 4, *B–D*). Interestingly, the floating cells from HT29 (Fig. 4*E*) and HCT15 cells (data not shown) showed evidence of a G2-M accumulation after 24 h. Forty-eight hours, *i.e.,* 24 h post drug removal, the floating HT29 cells had G_1–sub-G_1 distribution (Fig. 4*E*). In contrast, floating HCT116 cells had a sub-G_1 distribution at all time points analyzed.

With a few exceptions, activation of apoptotic proteases (caspases) is a key event required for the induction of apoptosis (16). Therefore, to confirm the induction of apoptosis by 17AAG treatment, we analyzed proteolytic activation of caspase 3, a critical executioner caspase, and cleavage of PARP, a caspase substrate (16). Fig. 5*A* shows that there was minimal cleavage of PARP in the control and treated adherent HT29 or HCT116 cell fractions at 72 h. In the small control floating cell fraction, ∼50% of PARP was cleaved. In contrast, PARP was present almost exclusively in the cleaved form in the floating population following 17AAG treatment of HCT116 cells and was increased in the floating population following treatment of HT29 cells (Fig. 5*A*). In the floating population of HT29 and HCT15 cells, the 17-kDa subunit produced following proteolytic activation of caspase 3 was detected only in the 17AAG-treated cells. In contrast, caspase 3 cleavage could not be detected in 17AAG-treated KM12 cells (Fig. 5*B*).

The small increase in floating cells and the absence of caspase cleavage following 17AAG treatment of KM12 cells suggested that, in contrast to the other three cell lines, 17AAG could not effectively induce apoptosis in this cell line. However, KM12 cells were not intrinsically defective in the induction of apoptosis: treatment with a

Table 1 *Summary of proteins affected after 24-h treatment with 17AAG*

Concentrations used were the minimum isoeffective dose sufficient to deplete c-Raf-1 to <95% of the untreated control at 24 h: 0.5 μM for HT29, 1 μM for HCT116 and KM12, and 50 μM for HCT15 cells. +/−, <95% of control; +, <50% of control; ++, equal to control; ND, not detected. All results were replicated in two independent experiments.

	c-Raf-1	Ki-ras	N-ras	ERK-1/2	Phospho-Erk-1/2	c-Akt	GAPDH
HT29							
Control	++	++	++	++	++	++	++
17AAG							
24 h	+/−	+	+	++	+	+/−	++
48 h	+/−	+/−	+/−	+/−	+/−	+/−	++
72 h	+/−	+/−	+/−	+	+	+	++
HCT116							
Control	++	++	++	++	++	++	++
17AAG							
24 h	+/−	+	+/−	+	+/−	+	++
48 h	++	+	++	++	+	+/−	++
72 h	++	+	++	++	+	++	++
KM12							
Control	++	++	++	++	ND	++	++
17AAG							
24 h	+	+	+	++	ND	+	++
48 h	+	+	+/−	+/−	ND	+	++
72 h	+	+	+	++	ND	++	++
HCT15							
Control	++	++	++	++	++	++	++
17AAG							
24 h	+/−	++	++	++	+	+/−	++
48 h	+	+	+	++	+	+	++
72 h	+	+	+	++	++	++	++

ability of 17AAG to induce apoptosis. On the basis of published studies and our data herein, we analyzed the expression of Bag-1, an antiapoptotic protein that interacts with Hsp70, and Bad, a proapoptotic protein that is phosphorylated by c-Akt and appears to link signal transduction and apoptotic pathways (17–22). Both of these proteins interact with Bcl-2 and will influence its ability to block the activity of proapoptotic proteins such as Bax (17, 18). Bcl-2 expression could barely be detected in all of the cell lines compared with the positive control (Fig. 5*C*). Expression of Bag-1 was significantly elevated in KM12 cells compared with the other colon cancer cell lines; in contrast, Bax was expressed in all cell lines except KM12 cells (Fig. 5*C*). Expression of Bad was relatively consistent across all of the cell lines (Fig. 5*C*). There were no consistent changes in the expression of any of these Bcl-2 family members following treatment with 17AAG (data not shown).

Murine Bad is phosphorylated on Ser-112 and Ser-136 by mitochondria-anchored protein kinase A, c-Akt, c-Raf-1, and Rsk-1 (17, 19–22). Phosphorylation of Bad results in sequestration by 14-3-3 proteins, blocking the proapoptotic function of Bad (17). Dephosphorylated Bad induces apoptosis by binding to and inhibiting the antiapoptotic effects of Bcl-2 (17). Phosphorylated Bad was immunoprecipitated with antibodies specific for the phosphoserine-112 or phosphoserine-136 sites. However, we could not detect any significant changes in phosphorylation at either site following treatment of HCT116 or KM12 cells with 17AAG (Fig. 5*D*).
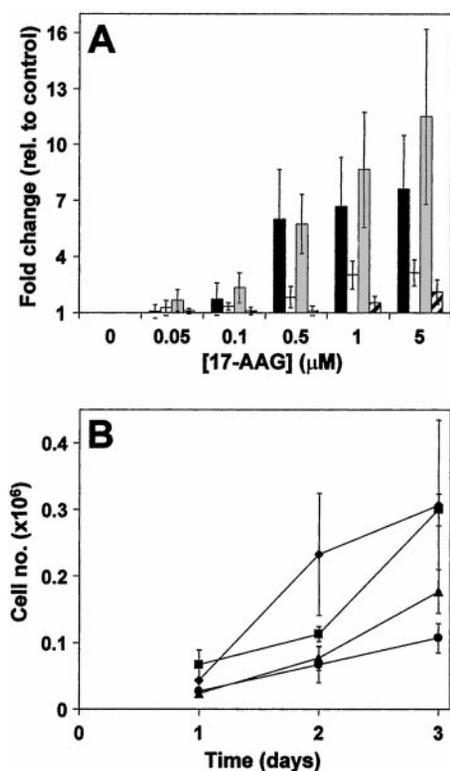

Fig. 3. HCT116 (■), HCT15 (▦), HT29 (□), and KM12 (▨) cells were treated with a range of 17AAG concentrations for 24 h and then cultured in the absence of 17AAG for an additional 48 h. *A*, floating cells relative to untreated controls (*n* = 3). *B*, detection of floating cells over a 72-h time course following treatment of HT29 (■), HCT116 (◆), KM12 (●), and HCT15 (▲) cells with 0.5, 1, 1, and 50 μM 17AAG, respectively. Results are mean values ± SE (*bars*) of mean of three independent experiments.

Fig. 4. *A*, typical morphology of control and 17AAG-treated HT29 and HCT116 adherent cells at 24 h and of 17AAG-treated HT29 and HCT116 floating cells at 72 h. *B–D*, cell cycle analysis of adherent cells following treatment of HT29, HCT116, KM12, and HCT15 cells with 0.5, 1, 1, and 50 μM 17AAG, respectively: *B*, untreated controls at 24 h; *C*, 17AAG-treated cells at 24 h; and *D*, 17AAG-treated cells at 48 h. *E*, cell cycle analysis of floating HT29 and HCT116 cells treated with 0.5 and 1 μM 17AAG, respectively. *d*, day. All results were duplicated in independent experiments.

pharmacologically relevant dose of etoposide (1 μM) did induce apoptosis (data not shown). One potential explanation was differential expression of members of the Bcl-2 family of cell death regulators (17). Because there are many members of this family, we chose in this initial study to examine selected members that might influence the
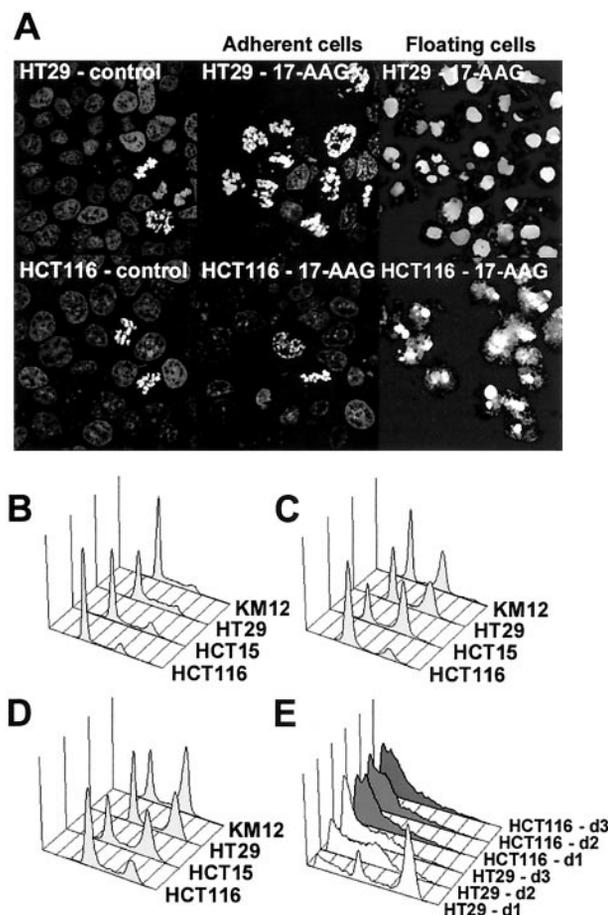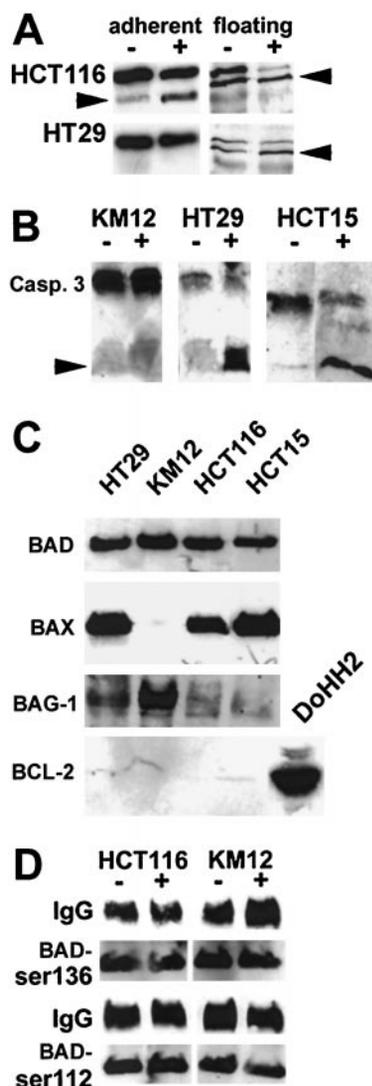
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Fig. 5. 17AAG and molecular determinants of apoptosis. *A*, cleavage of PARP at 72 h in HCT116 and HT29 adherent and floating cell populations following treatment with 1 or 0.5 μM 17AAG (*arrow* indicates cleavage product). *B*, cleavage of caspase 3 at 72 h in the floating population of KM12, HT29, and HCT15 cells treated with 1, 0.5, and 50 μM 17AAG, respectively. *C*, characterization of Bad, Bax, Bag-1, and Bcl-2 expression in untreated HT29, KM12, HCT116, and HCT15 cells. DoHH2 non-Hodgkin's lymphoma cells were included as a positive control for Bcl-2 expression. *D*, phosphorylation of Bad in HCT116 and KM12 cells following 24-h treatment with 1 μM 17AAG. Phosphorylated Bad was immunoprecipitated with an antibody specific to the phosphorylated Ser-136 or Ser-112 sites and then detected by Western blotting with an antibody that recognizes both phosphorylated and nonphosphorylated Bad. All blots were duplicated in at least two independent experiments.

DISCUSSION

17AAG has potent antitumor activity and recently entered a Phase I study, under the auspices of NCI and CRC, at our center and other sites in the United States. This agent inhibits the molecular chaperone function of Hsp90, an activity that is crucial for maintaining stability and function of several key oncogenic signaling molecules such as c-Raf-1 and Src kinases. As far as we are aware, 17AAG is the first Hsp90 inhibitor to enter clinical study. Although there is strong evidence that Hsp90 is the primary target of 17AAG, the downstream biochemical and cellular consequences of 17AAG treatment may vary with the molecular makeup of different tumors. We previously showed that 17AAG inhibited the growth of human colon adenocarcinoma cells *in vitro* and of corresponding xenograft tumors *in vivo*; however, the client proteins and signaling pathways affected by 17AAG were not well defined in this cell type (3). Colon cancer is of

particular interest with respect to 17AAG because of the high incidence of *Ki-ras* mutations, the constitutive activation of Ras/Raf/MEK/ERK, and the sensitivity to specific inhibitors of this signal transduction pathway (11–13). The present studies were aimed at providing a better understanding of the mechanism of action of 17AAG in human colon cancer by studying four different human colon cancer cell lines, chosen from members of the NCI cell line panel, whose gene expression profile in response to 17AAG was recently determined (14). Of particular interest was the identification of additional proteins that were depleted or affected downstream of Hsp90 inhibition and the cellular sequelae, particularly in terms of cytostasis and apoptosis.

We demonstrated a range of clear and readily replicated effects on important oncogenic proteins, the precise details of which varied in a reproducible manner between different colon cancer cell lines. We confirmed the depletion of c-Raf-1 and inhibition of ERK-1/2 phosphorylation seen previously with 17AAG (3, 10). We have also demonstrated here that c-Akt levels are depleted and c-Akt phosphorylation is inhibited by 17AAG. c-Akt is pivotal in a number of survival pathways, including the PI3 kinase pathway and signaling through nuclear factor-κB (23). Our observation is consistent with c-Akt depletion in DLD-1 human colon cancer cells by the related ansamycin, herbimycin A (24). Interestingly, we also noted that Ki-ras and N-ras were depleted by 17AAG, an effect not apparently reported previously for this drug class. Depletion of Ki-ras may be particularly important in colorectal cancer where *Ki-ras* gene mutations are common and associated with poor outcome (11). Concentrations required to deplete these key signaling molecules were close to the IC_{50} doses for adherent cell viability at 72 h. Thus, 17AAG has wide-ranging effects on key oncogenic signaling components that regulate both proliferation and survival at doses that are pharmacologically relevant.

At the cellular level we showed that treatment of human colon cancer cells with 17AAG resulted in cytostasis and, with the exception of HCT116 cells, the accumulation of mitotic figures that was consistent with a G_2-M cell cycle delay. This was confirmed by flow cytometry and was consistent with observations that herbimycin A and geldanamycin also induce G_2-M arrest in a colon and an ovarian cancer cell line, respectively (25). A recent study has proposed that herbimycin A induces a G_1 cell cycle block that is dependent on the Rb gene product, whereas cells that lacked the Rb gene product became arrested at the G_2-M phase of the cell cycle (26). However, deletion or loss of expression of Rb is rare in colon cancer and overexpression is common (27). Unlike the aforementioned study (26), we detected G_2-M arrest in three of the four cell lines (HT29, HCT15, and KM12) despite Rb expression in all of them (27, 28).

HCT116 cells did not experience G_2-M arrest. In addition to expressing Rb protein, this cell line also has wild-type p53. Reduction of the ansamycin benzoquinone ring could yield reactive oxygen species that damage DNA. Therefore, p53 status may influence cell cycle arrest following 17AAG treatment. However, we could not detect induction of p53-responsive genes following 17AAG treatment of HCT116 cells (14). Yeast mutants with a mutated heat shock transcription factor 1, which is responsive to Hsp90 inhibition, undergo G_2-M arrest and have reduced levels of Hsp90 (29). This effect was suppressed by Hsp90 expression, implying that Hsp90 or presumably a specific Hsp90 client was required for G_2-M progression. This suggests that G_2-M arrest is primarily the result of depletion of a key Hsp90 client, whereas the reasons for the absence of a G_2-M block in HCT116 cells remain unclear.

In addition to cell cycle arrest, we also demonstrated, to our knowledge for the first time, that 17AAG induces apoptosis. This was shown by morphological analysis and confirmed by proteolytic cleavage of PARP and caspase 3. Of note, 17AAG induced apoptosis in

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HT29, HCT116, and HCT15 cells (although the kinetics were different in each case), but not in the KM12 line. KM12 and HCT116 cells had a similar IC_{50} for adherent cell viability, and there were no apparent differences in the client proteins depleted by 17AAG between the two cell lines. KM12 cells did undergo apoptosis in response to etoposide, a topoisomerase II inhibitor that produces DNA damage. Etoposide reportedly induces apoptosis through two proapoptotic proteins, Bax and Bak, or by activation of the Fas pathway (30–32). Our observations suggest that although KM12 cells are competent to undergo apoptosis, 17AAG uses pathways different from those used by etoposide.

Induction of an heat shock factor-1-dependent stress response can protect cells from 17AAG (33). We found that a short exposure of 45 min at 44°C resulted in a limited induction (2-fold) of Hsp70 in all four cell lines, whereas a milder heat shock of 24 h at 42°C resulted in induction of Hsp70 in KM12 cells alone.[4] This suggests that active heat shock response in KM12 might be responsible for protecting the cells against the induction of apoptosis, but not cytostasis. However, in contrast to the primary cells analyzed by Bagatell *et al.* (33), but in common with many cancer types (34), all four colon cancer cell lines expressed considerable constitutive levels of Hsp70 that may mask any further induction by heat shock.

The antiapoptotic Bag-1 protein binds Hsp70, whereas the proapoptotic Bad protein is a substrate of c-Akt and a number of other survival signaling pathways (17–22). Both of these proteins can also bind the antiapoptotic Bcl-2 protein and will influence its ability to block the action of proapoptotic proteins such as Bax (17). Compared with the other cell lines, KM12 cells had barely detectable expression of Bax, but exhibited a clear and reproducibly higher level of Bag-1. We hypothesized that given the depletion of the key signaling molecules that phosphorylate and regulate Bad, a consequence of 17AAG treatment should be dephosphorylation of Bad and the induction of apoptosis (17, 19–22). However, we saw no change in Bad phosphorylation. One explanation could be that Bad phosphorylation is maintained by a kinase unaffected by Hsp90 inhibition. Alternatively, the phosphatase required for Bad dephosphorylation may be depleted as an Hsp90 client. There is a precedent for this: calcineurin, a phosphatase that can induce apoptosis by dephosphorylating Bad, is also an Hsp90 client (7, 35). Signaling through c-Akt also blocks apoptosis induced by overexpression of a variety of proapoptotic proteins, including Bax, Bak, and Bik, and can protect cells from apoptotic stimuli in the absence of Bad expression (36). In addition, c-Akt phosphorylates and inactivates caspase 9, a key apoptotic protease required for initiating the cascade of proteases (37). Studies using gene transfer approaches are now necessary to further investigate whether Bax or Bag-1 can influence the apoptotic response to 17AAG. A more comprehensive analysis should also include the proapoptotic Bak protein and the Fas pathway, both of which may be activated following treatment with chemotherapeutic agents.

As discussed earlier, treatment with 17AAG results in the accumulation of mitotic figures. This could be attributable to prevention of Cdk activation via inhibition of ERK1/2 and PI3 kinase signal transduction pathways or alternatively may relate to the fact that tubulin is an Hsp90 client (8). Interestingly, analysis of drug activity alone or correlation of drug activity with array analysis of gene expression across the NCI panel of 60 tumor lines results in geldanamycin clustering in the same hierarchical node as tubulin inhibitors (38). COMPARE analysis of the NCI cell line panel data also suggests that geldanamycin and 17AAG share similarities with paclitaxel and vincristine (maximum COMPARE values of 0.712 and 0.639, respectively, for paclitaxel and 0.698 and 0.599, respectively, for vincris-

tine).[5] These agents affect the mitotic spindle and, interestingly, their cytotoxicity is enhanced by Bax expression (39). This raises the interesting possibility that the cellular outcome of 17AAG treatment involves either (*a*) cytostasis resulting from inhibition of the growth factor receptor Ras/Raf/MEK/ERK pathway or (*b*) apoptosis induced by the interruption of survival signaling pathways through c-Akt or by direct or indirect interference with the mitotic spindle.

Understanding the basis of the antitumor activity of 17AAG may be critical for its optimal evaluation and use in patients and for development of novel Hsp90 inhibitors. At the moment, the story is far from complete in terms of how the molecular and cellular effects of 17AAG reported here and elsewhere could be applied in the treatment of colorectal and other tumors. Some possibilities are, however, beginning to emerge. In some ways 17AAG is a unique anticancer agent that apparently has a single primary molecular target (6), but whose action results in effects on several important oncogenic proteins. For example, here we show evidence for inhibition of signaling through both the Ras/Raf/MEK/ERK and PI3 kinase/PDK/Akt pathways. The complexity of the response at the cellular and molecular level, demonstrated here by protein analysis and also by gene expression profiling (14), makes it difficult to predict at present how generally applicable our observations would be to different tumor types. We and others have demonstrated that 17AAG can deplete c-Raf-1 in ovarian, breast, and colon cancer cell lines and inhibit ERK-1/2 phosphorylation in breast and colon cancer cell lines (3, 10). It is likely that many of the individual proteins depleted by 17AAG and the signaling pathways inhibited will be similar across different cell types, albeit with differences in concentration dependence. However, events further downstream of this protein depletion will depend on how reliant the particular cell is on the proteins that are depleted by 17AAG and also on the expression profile of cell cycle control and pro- and antiapoptotic proteins within that cell type. For example, effects on Ki-ras signaling may be important in colorectal cancer, and depletion of erbB2 may be crucial in breast cancer.

In addition to the molecular parameters described above, previous studies showed that cellular response can be affected by expression of drug-metabolizing and -transport proteins, namely DT-diaphorase and P-glycoprotein (3). Expression profiling revealed that levels of the target Hsp90β mRNA and protein are influenced by 17AAG treatment (14). In some cells, *e.g.,* HCT116, 17AAG induces Hsp90β expression, whereas Hsp90β is decreased in other cell lines, such as HT29. Thus the molecular pharmacology of 17AAG is complex, and gene transfer studies are under way in our laboratory to elucidate the most important determinants of response in various tumor types. Each of the four colon cell lines had a unique gene expression profile in response to 17AAG (14). Understanding the key elements in the overall relationship between molecular and cellular response across colon and other tumor types may be helped by more extensive expression profiling experiments following 17AAG treatment, coupled with correlation of client proteins depleted and the biological consequences of 17AAG treatment.

Despite the potential complexities of the molecular pharmacology of 17AAG, we have shown here for the first time that both cytostatic and apoptotic events can be induced at concentrations close to the IC_{50} of the drug in human colon cancer cells and indeed at concentrations that are achieved in animal models and in plasma of patients treated in the Phase I trial.[6] Both cytostatic and apoptotic outcomes could contribute to any therapeutic effects that may be achieved with 17AAG in the clinic, and their relative involvement could impact on the optimal scheduling, *e.g.,* chronic dosing to maintain a cytostatic response or intermittent dosing to achieve tumor regression through

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[4] P. A. Clarke and F. DiStefano, unpublished observations.

[5] P. A. Clarke, unpublished observations, based on data at http://dtp.nci.nih.gov.

[6] F. Raynaud, CRC Center for Cancer Therapeutics, personal communication.

apoptosis. However, normal tissue tolerance will also impact on the clinical schedule. Should our observations of apoptotic and cytostatic outcomes prove relevant in the clinic, the use of existing assays for these effects might be possible. Apoptotic and cytostatic responses have been assessed *in situ* following treatment of breast cancer (40). However, performing frequent or repeat biopsies may prove to be impractical in colon and several other cancers, and the development of surgically noninvasive assays (*e.g.,* based on magnetic resonance or positron emission tomography technology) would be preferable. An alternative to direct assays of cytostasis *versus* apoptosis would be to use individual molecular markers that are predictive of these cellular consequences in response to 17AAG. These may be defined by extending the present analysis at the protein level. Expression profiling with correlation to biological outcome may allow the identification of single genes or clusters of markers that could be used to screen pretreatment biopsies for prediction of the type of response (14).

In conclusion, cytostatic *versus* apoptotic responses to 17AAG may vary in individual tumors, and a more detailed understanding of the mechanisms involved may provide molecular determinants that could be useful in the clinic. Our results also point to molecular mechanisms that can be pursued by gene transfer strategies to determine causation (particularly the involvement of Bax and Bag-1) and highlight parameters that might be analyzed in tumor biopsies taken during ongoing Phase I and any future Phase II studies.

ACKNOWLEDGMENTS

REFERENCES

1. Garrett, M., and Workman, P. Discovery of new chemotherapeutic drugs for the third millennium. Eur. J. Cancer, *35:* 2010–2030, 1999.
2. Gelmon, K., Eisenhauer, E., Harris, A., Ratain, M., and Workman, P. Anticancer agents targeting signaling molecules and cancer cell environment: challenges for drug development? J. Natl. Cancer Inst. (Bethesda), *91:* 1281–1287, 1999.
3. Kelland, L. R., Sharp, S. Y., Rogers, P. M., Myers, T. G., and Workman, P. DT-diaphorase expression and tumor cell sensitivity to 17-allyamino,17-demethoxygeldanamycin, an inhibitor of heat shock protein 90. J. Natl. Cancer Inst. (Bethesda), *91:* 1940–1949, 1999.
4. Uehara, Y., Hori, M., Takeuchi, T., and Umezawa, H. Screening of agents which convert 'transformed morphology': identification of an active agent as herbimycin and its inhibition of intracellular src kinase. Jpn. J. Cancer Res., *76:* 672–675, 1985.
5. Supko, J. G., Hickman, R. L., Grever, M. R., and Malspeis, L. Preclinical pharmacological evaluation of geldanamycin as an antitumor agent. Cancer Chemother. Pharmacol., *36:* 305–315, 1995.
6. Neckers, L., Schulte, T. W., and Mimnaugh, E. Geldanamycin as a potential anticancer agent: its molecular target and biochemical activity. Investig. New Drugs, *17:* 361–373, 1999.
7. Brunton, V. G., Steele, G., Lewis, A. D., and Workman, P. Geldanamycin-induced cytotoxicity in human colon-cancer cell lines: evidence against the involvement of c-Src or DT-diaphorase. Cancer Chemother. Pharmacol., *41:* 417–422, 1998.
8. Buchner, J. Hsp90 & Co.—a holding for folding. Trends Biochem. Sci., *24:* 136–141, 1999.
9. Schnur, R. C., Corman, M. L., Gallaschun, R. J., Cooper, B. A., Dee, M. F., Doty, J. L., Muzzi, M. L., Moyer, J. D., DiOrio, C. I., Barbacci, E. G., Millar, P. E., O'Brien, A. T., Morin, M. J., Foster, B. A., Pollack, V. A., Savage, D. M., Sloan, D. E., Pustilnik, L. R., and Moyer, M. P. Inhibition of the oncogene product p185erbB-2 *in vitro* and *in vivo* by geldanamycin and dihydrogeldanamycin derivatives. J. Med. Chem., *38:* 3806–3812, 1995.
10. Schulte, T. W., and Neckers, L. M. The benzoquinone ansamycin 17-allylamino-17-demethoxygeldanamycin binds to Hsp90 and shares important biologic activities with geldanamycin. Cancer Chemother. Pharmacol., *42:* 273–279, 1998.
11. Andreyev, H. J., Norman, A. R., Cunningham, D., Oates, J. R., and Clarke, P. A. Kirsten ras mutations in patients with colorectal cancer: the multicenter "RASCAL" study. J. Natl. Cancer Inst. (Bethesda), *90:* 675–684, 1998.
12. Hoshino, R., Chatani, Y., Yamori, T., Tsuruo, T., Oka, H., Yoshida, O., Shimada, Y., Ari, I-S., Wada, H., Fujimoto, J., and Kohno, M. Constitutive activation of the 41–43-kDa mitogen-activated protein kinase signalling pathway in human tumors. Oncogene, *18:* 813–822, 1999.
13. Sebolt-Leopold, J. S., Dudley, D. T., Herrera, R., Van Becelaere, K., Wiland, A., Gowan, R. C., Tecle, H., Barrett, S. D., Bridges, A., Przybranowski, S., Leopold, W. R., and Saltiel, A. R. Blockade of the MAP kinase pathway suppresses growth of colon tumors *in vivo*. Nat. Med., *5:* 810–816, 1999.
14. Clarke, P. A., Hostein, I., Banerji, U., Di Stefano, F., Maloney, A., Walton, M., Judson, I., and Workman, P. Gene expression profiling of human colon adenocarcinoma cells following inhibition of signal transduction by 17-allylamino-17-demethoxygeldanamycin, an inhibitor of the Hsp90 molecular chaperone. Oncogene, *19:* 4125–4133, 2000.
15. Fitzsimmons, S. A., Workman, P., Grever, M., Paull, K., Camalier, R., and Lewis, A. D. Reductase enzyme expression across the National Cancer Institute Tumor cell line panel: correlation with sensitivity to mitomycin C and EO9. J. Natl. Cancer Inst. (Bethesda), *88:* 259–269, 1996.
16. Zheng, T. S., Hunot, S., Kuida, K., and Flavell, R. A. Caspase knockouts: matters of life and death. Cell. Death Differ., *6:* 1043–1053, 1999.
17. Korsmeyer, S. J. Bcl-2 gene family and the regulation of programmed cell death. Cancer Res., *59:* 1693–1700, 1999.
18. Stuart, J. K., Myszka, D. G., Joss, L., Mitchell, R. S., McDonald, S. M., Xie, Z., Takayama, S., Reed, J. C., and Ely, K. R. Characterisation of interactions between the anti-apoptotic protein bag-1 and hsc70 molecular chaperones. J. Biol. Chem., *273:* 22506–22514.
19. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. Akt phosphorylation of Bad couples survival signals to the cell-intrinsic death machinery. Cell, *91:* 231–241, 1997.
20. Harada, H., Becknell, B., Wilm, M., Mann, M., Jun-shen, L., Taylor, S. S., Scott, J. D., and Korsmeyer, S. J. Phosphorylation and inactivation of Bad by mitochondrially-anchored protein kinase A. Mol. Cell, *3:* 413–422, 1999.
21. Wang, H-G., Rapp, U. R., and Reed, J. C. Bcl-2 targets the protein kinase raf-1 to mitochondria. Cell, *87:* 629–638, 1996.
22. Shimamura, A., Ballif, B. A., Richards, S. A., and Blenis, J. Rsk1 mediates a MEK-MAP kinase cell survival signal. Curr. Biol., *10:* 127–135, 2000.
23. Romashkova, J. A., and Makarov, S. S. NF-κB is a target of akt in antiapoptotic PDGF signalling. Nature (Lond.), *401:* 86–89, 1999.
24. Muise-Helmericks, R. C., Grimes, H. L., Bellacosa, A., Malstrom, S. E., Tsichlis, P. N., and Rosen, N. Cyclin D expression is controlled post-transcriptionally via phosphatidylinositol 3-kinase/Akt-dependent pathway. J. Biol. Chem., *273:* 29864–29872, 1998.
25. Mancini, M., Anderson, B. O., Caldwell, E., Sedhinasab, M., Paty, P. B., and Hockenberry, D. M. Mitochondrial proliferation and paradoxical depolarization during terminal differentiation and apoptosis in a human colon carcinoma cell line. J. Cell. Biol., *138:* 449–469, 1997.
26. Srethapakdi, M., Liu, F., Tavorath, R., and Rosen, N. Inhibition of Hsp90 function by ansamycins causes retinoblastoma gene product-dependent G_1 arrest. Cancer Res., *60:* 3940–3946, 2000.
27. Wildrick, D. M., and Boman, B. M. Does the human retinoblastoma gene have a role in colon cancer? Mol. Carcinog., *10:* 1–7, 1994.
28. Ali, A. A., Marcus, J. N., Harvey, J. P., Roll, R., Hodgson, C. P., Wildrick, D. M., Chakraborty, A., and Boman, B. M. RB1 protein in normal and malignant human colorectal tissue and colon cancer cell lines. FASEB J., *7:* 931–937, 1993.
29. Morano, K. A., Santoro, N., Koch, K. A., and Thiele, D. J. A trans-activation domain in yeast heat shock transcription factor is essential for cell cycle progression during stress. Mol. Cell Biol., *19:* 402–411, 1999.
30. Micheau, O., Solary, E., Hammann, A., and Dimanche-Boitrel, M. T. Fas ligand-independent. FADD-mediated activation of the Fas death pathway by anticancer drugs. J. Biol. Chem., *274:* 7987–7992, 1999.
31. Simonian, P. L., Grillot, D. A., and Nunez, G. Bak can accelerate chemotherapy-induced cell death independently of its heterodimerization with Bcl-XL and Bcl-2. Oncogene, *15:* 1871–1875, 1997.
32. McCurrach, M. E., Connor, T. M., Knudson, C. M., Korsmeyer, S. J., and Lowe, S. W. Bax-deficiency promotes drug resistance and oncogenic transformation by attenuating p53-dependent apoptosis. Proc. Natl. Acad. Sci. USA, *94:* 2345–2349, 1997.
33. Bagatell, R., Paine-Murieta, G. D., Taylor, C. W., Pulcini, E. J., Akinaga, S., Benjamin, I. J., and Whitesell, L. Induction of a heat shock factor 1-dependent stress response alters the cytotoxic activity of Hsp90-binding agents. Clin. Cancer Res., *6:* 3312–3318, 2000.
34. Jaettela, M. Escaping cell death: survival proteins in cancer. Exp. Cell. Res., *248:* 30–43, 1999.
35. Wang, H-G., Pathan, N., Ethell, I. M., Krajewski, S., Yamaguchi, Y., Shibasaki, F., McKeon, F., Bobo, T., Franke, T. F., and Reed, J. C. Ca^{2+}-induced apoptosis through calceneurin dephosphorylation of Bad. Science (Wash. DC), *284:* 339–343, 1999.
36. Kennedy, S. G., Kandel, E. S., Cross, T. K., and Hay, N. Akt/protein kinase B inhibits cell death by preventing the release of cytochrome c from mitochondria. Mol. Cell. Biol., *19:* 5800–5810, 1999.
37. Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. Regulation of cell death protease caspase-9 by phosphorylation. Science (Wash. DC), *282:* 1318–1321, 1998.
38. Scherf, U., Ross, D. T., Waltham, M., Smith, L. H., Lee, J. K., Tanabe, L., Kohn, K. W., Reinhold, W. C., Myers, T. G., Andrews, D. T., Scudiero, D. A., Eisen, M. B., Sausville, E. A., Pommier, Y., Botstein, D., Brown, P. O., and Weinstein, J. N. A gene expression database for the molecular pharmacology of cancer. Nat. Genet., *24:* 236–244, 2000.
39. Strobel, T., Swanson, L., Korsmeyer, S., and Cannistra, S. A. Bax enhances paclitaxel-induced apoptosis through a p53-independent pathway. Proc. Natl. Acad. Sci. USA, *93:* 14094–14099, 1996.
40. Ellis, P. A., Smith, I. E., McCarthy, K., Detre, S., Salter, J., and Dowsett, M. Preoperative chemotherapy induces apoptosis in early breast cancer. Lancet, *349:* 849, 1997

Inhibition of Signal Transduction by the Hsp90 Inhibitor 17-Allylamino-17-demethoxygeldanamycin Results in Cytostasis and Apoptosis

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