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ORIGINAL ARTICLE

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The benzoquinone ansamycin 17-allylamino-17-demethoxygeldanamycin binds to HSP90 and shares important biologic activities with geldanamycin

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Abstract *Purpose*: Benzoquinone ansamycins are antibiotics with anticancer potential. First described as tyrosine kinase inhibitors, they are now frequently used to target HSP90 chaperone function. While herbimycin A and geldanamycin (GA) have been widely used in preclinical studies, both drugs are poor candidates for clinical trials owing to their in vivo toxicity and lack of stability. We therefore examined the biologic effects of 17-allylamino-17-demethoxygeldanamycin (17-AG), an ansamycin derivative with lower in vivo toxicity than GA. *Methods*: Binding of 17-AG to HSP90 was studied in vitro using a GA-affinity beads competition assay. We analyzed the drug-induced destabilization of p185erbB2, Raf-1 and mutant p53 in SKBR3 breast cancer cells by Western blotting. The antiproliferative activities of 17-AG and GA were compared using the MTT assay. *Results*: We found that, in a similar manner to GA itself, 17-AG bound specifically to HSP90. It also led to degradation of the receptor tyrosine kinase $p185^{erbB2}$, the serine/threonine kinase Raf-1 and mutant p53. Both GA and 17-AG displayed comparable antiproliferative effects in SKBR3 and MCF7 cells. Even though HSP90 binding by 17-AG was weaker than by GA, 17-AG and GA caused biologic effects in tumor cells at similar doses. *Conclusion*: 17-AG shares the important biologic features of its parent compound GA. Since 17-AG has a better toxicity profile than GA, it is an interesting candidate benzoquinone ansamycin for clinical development.

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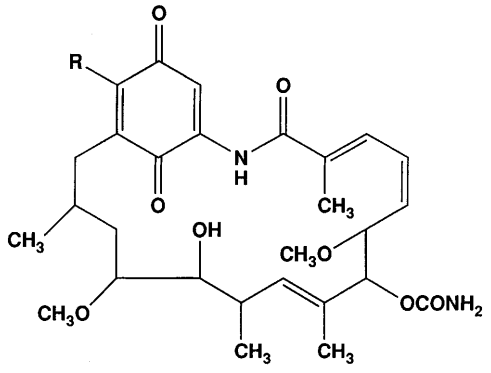
Introduction

The benzoquinone ansamycins herbimycin A (HA), geldanamycin (GA) and macbecin belong to a class of antibiotics that was first isolated from actinomycete broth [8, 20]. The biologic relevance of this class of chemicals became evident, when HA was shown to cause the morphology of Rous sarcoma virus-transformed fibroblasts to revert to a normal phenotype [31, 32]. After HA was found to inhibit the activity of other protein tyrosine kinases such as yes, $p185^{erbB2}$ and abl [14, 19, 30, 33], HA became a frequently used general tyrosine kinase inhibitor.

A study of the mechanism of action of the effect of GA on v-src has revealed that GA binds to the heat-shock protein HSP90 [36]. Newly synthesized v-src exists in a multimolecular complex with HSP90 while it is moving to the plasma membrane [5, 6]. At low concentrations, GA causes disruption of this multimolecular complex [36]. Subsequently, other cellular proteins that interact with HSP90 have been found to be affected by GA, such as steroid hormone receptors [10, 28, 35], mutant p53 [3], Raf-1 [23] and Cdk4 [27]. $P185^{erb\text{-}B2}$, which is rapidly degraded after ansamycin treatment [14], forms a complex with GRP94, a chaperone protein highly homologous with HSP90. GRP94, which is important for $p185^{erb\text{-}B2}$ processing, is also targeted by GA [7].

Benzoquinone ansamycins have been found to have antitumor activity against cancer cell lines and in animal models. In the National Cancer Institute's (NCI) in vitro screen of drug sensitivity in 60 tumor cell lines, GA achieved 50% growth inhibition at 13 n*M* in highly responsive cell lines with an overall mean of 180 n*M* [29]. However, the development of GA as a clinical agent has so far been limited by its toxicity, especially liver toxicity [29]. Because of the promising antitumor properties of



GA: R= CH_3O

17-AG: R= $H_2C{=}CHCH_2NH$

Fig. 1 Structures of the benzoquinone ansamycins GA and 17-AG

the benzoquinone ansamycins, the instability of HA and the toxicity of GA, the development of biologically active derivatives has become an interesting and important endeavor.

17-Allylamino-17-demethoxygeldanamycin (17-AG, Fig. 1) has shown high activity in a screen against $p185^{erb\text{-}B2}$ [22]. It compares favorably with GA in terms of animal toxicity and causes fewer hepatic side effects [21]. We now report that 17-AG binds to HSP90 and, together with GA, shows effects on several important cellular proteins, including $p185^{erb\text{-}B2}$, Raf-1 and mutant p53. Our results indicate that 17-AG appears to be a good candidate for further clinical development.

Materials and methods

Materials

GA and 17-AG were obtained from the Developmental Therapeutics Program, NCI (Rockville, Md.). The drugs were dissolved in DMSO as 5 m*M* stock solutions. The antibodies used were mouse monoclonal HSP90 antibody (clone MA3-011, Affinity BioReagents, Neshanic Station, N.J.), mouse monoclonal antibody 3 for $p185^{erb\text{-}B2}$ (clone 3B5, Oncogene Science, Uniondale, N.Y.), rabbit polyclonal Raf-1 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.), phosphospecific MAPK antibody (New England Biolabs, Beverly, Mass.) and anti-p53 monoclonal antibody PAb1801 Oncogene Science). All other chemicals were of the highest analytical grade.

Cell culture

SKBR3 and NIH3T3 cells were obtained from the American Type Culture Collection (Rockville, Md.). MCF7 cells were provided by Dr. K. Cowan (NCI, Bethesda, Md.). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum and 10 m*M* HEPES.

Western blotting

Cells were lysed with TNES buffer (50 m*M* Tris-HCl, pH 7.5, 1% NP40, 2 m*M* EDTA, 100 m*M* NaCl) containing 1 m*M* sodium orthovanadate, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 m*M* PMSF, 25 m*M* NaF and 25 m*M* β-glycerophosphate. Total protein (25 μg) was separated on 8% SDS-polyacrylamide gels, transferred to nitrocellulose membrane by electroblotting and probed with the indicated primary antibodies as previously described [23]. We used horseradish peroxidase (HRP)-conjugated secondary antibody to rabbit or mouse IgG (Amersham, Arlington Heights, Ill.) in conjunction with Western blot chemiluminescence reagent (Renaissance, Du Pont, Wilmington, Del.). Films were scanned into a Macintosh computer using a Foto/Eclipse Gel Analysis System (Fotodyne, Hartland, Wis.) and processed using Adobe Photoshop software.

Production of GA-affinity beads

GA was derivatized and immobilized as previously reported [36]. Briefly, 1,6-hexanediamine was added to GA (10 m*M* in $CHCl_3$) at a tenfold molar excess and allowed to react for 2 h. After aqueous extraction, 17-hexamethylenediamine-17-demethoxygeldanamycin was dried, redissolved in DMSO and reacted with AffiGel 10 resin (Bio-Rad, Hercules, Calif.). The resulting beads were washed in TNES buffer and blocked in 1% bovine serum albumin before use.

Antiproliferative assay

SKBR3 or MCF7 cells were seeded in 96-well plates at a density of 3000 cells per well. The cells were treated 24 h later in quadruplicate with increasing doses of GA or 17-AG as indicated. Numbers of viable cells were assessed using the MTT assay after 4 days, as described previously [1].

Results

17-AG binds HSP90

NIH3T3 cells were lysed with TNES buffer and lysates were incubated with GA-affinity beads. After washing the beads, precipitated proteins were analyzed by silver staining (Fig. 2A) and immunoblotting with HSP90 antibody (Fig. 2B). Two bands were detected by silver staining, one at 90 kDa which represents HSP90 and another at 68 kDa which represents bovine serum albumin, which was used to block the beads (Fig. 2A). A competition assay was used to assess binding of 17-AG to HSP90. Both GA and 17-AG effectively competed with solid phase GA for binding to HSP90, but higher concentrations of 17-AG were required to achieve this effect compared with the parent compound (EC_{50} values for competition were 0.17 μ*M* for GA and 7.2 μ*M* for 17-AG, Fig. 2C). Therefore, 17-AG appeared to have a lower affinity for HSP90 than GA.

17-AG depletes cells of $p185^{erbB2}$

The human breast carcinoma cell line SKBR3 expresses a high level of $p185^{erbB2}$. SKBR3 cells were treated with increasing doses of 17-AG for 6 h. Protein levels were assessed by immunoblotting. The levels of $p185^{erbB2}$

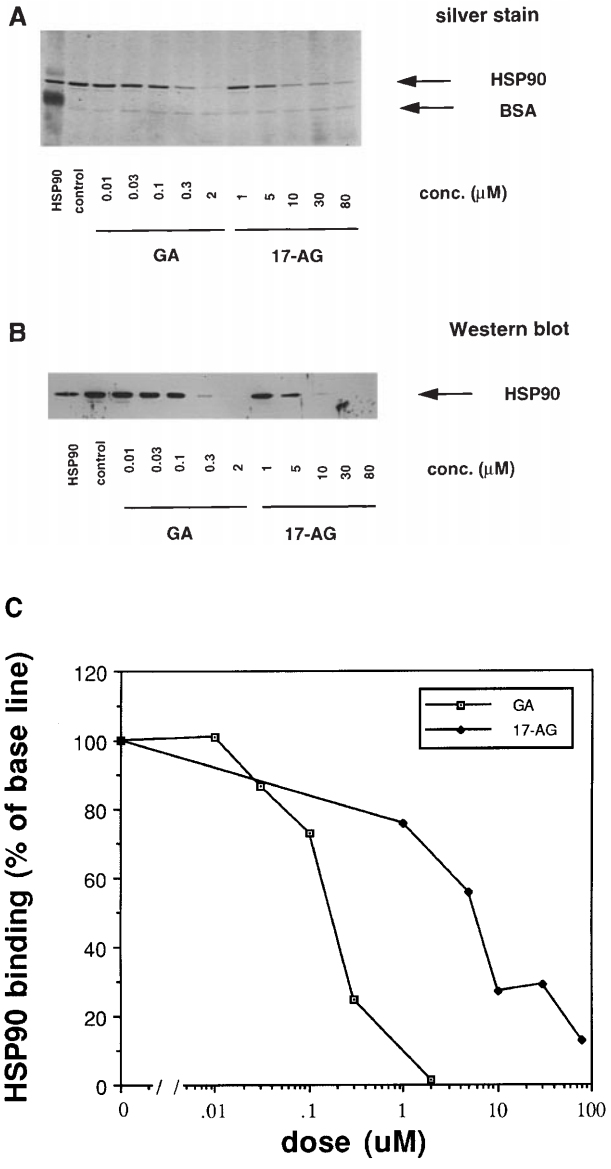


Fig. 2A–C 17-AG competes with GA for HSP90 binding. Lysates were prepared from SKBR3 cells and incubated with GA-affinity beads. After washing the affinity-precipitated proteins were separated on 8% SDS PAGE gels and analyzed by silver staining (**A**) or Western blotting with HSP90 antibody (**B**). Binding of HSP90 to GA affinity beads was competitively blocked by increasing doses of soluble GA or 17-AG. **C** Bands were measured by densitometry and plotted against dose

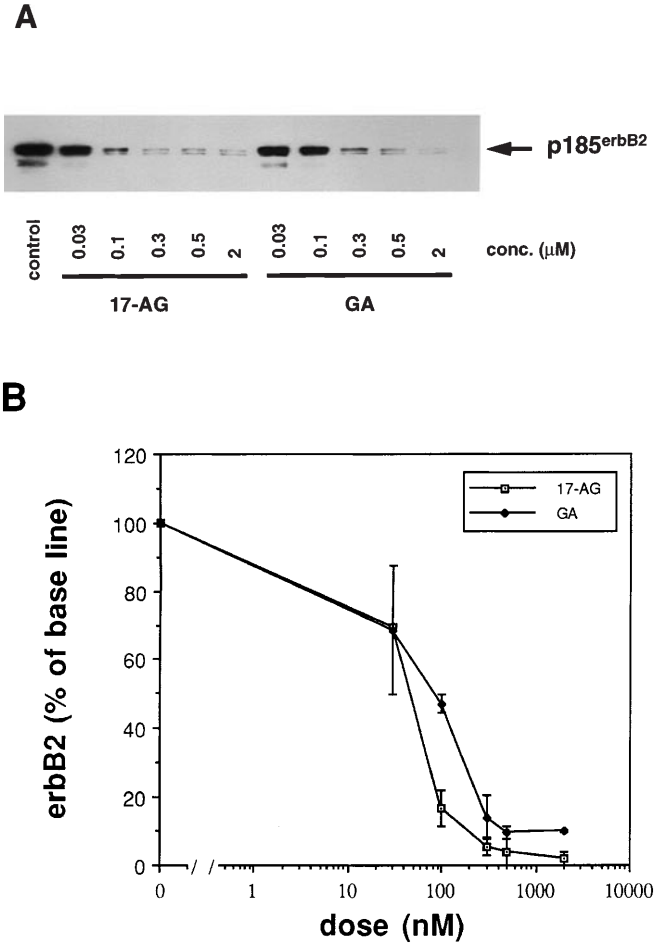


Fig. 3A,B 17-AG depletes cells of $p185^{erbB2}$ in a dose-dependent fashion. SKBR3 cells were treated for 6 h with increasing doses of 17-AG or GA. Lysates of treated cells were analyzed by Western blotting. **A** Western blot of one of three experiments; **B** results of densitometry of three experiments

were significantly reduced by 17-AG treatment and the EC_{50} was 45 n*M* (Fig. 3). The EC_{50} for GA was 90 n*M*.

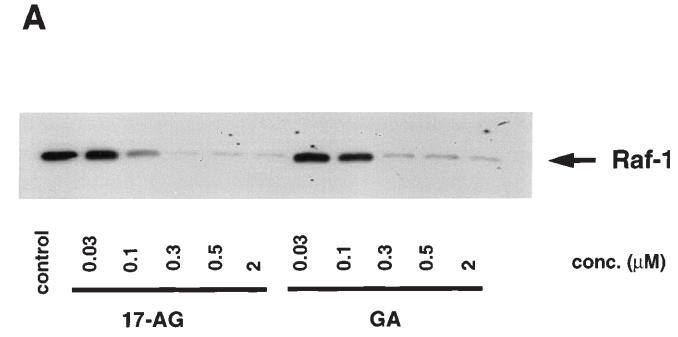
Destabilization of Raf-1 by 17-AG

Next we treated SKBR3 cells with increasing doses of 17-AG for 16 h and lysed the cells in TNES buffer. Raf-1 levels were significantly decreased upon drug treatment as assayed by Western blotting (Fig. 4). The EC_{50} values for 17-AG and GA were 80 and 170 n*M*, respectively. There was also a decrease in Raf-1 levels in NIH3T3 and MCF7 cells (data not shown).

Raf-1 serves as a part of the Raf-1-MEK-MAPK pathway [13, 16]. We analyzed the function of this pathway by stimulating NIH3T3 cells with PMA and measured the phosphorylation of MAPK with a phospho-MAPK-specific antibody [24]. 17-AG pretreatment 16 h prior to PMA stimulation inhibited the increase in phosphorylated MAPK as did the parent compound GA (Fig. 5). The dose used was 2 μ*M*, which resulted in a more than 90% decrease in Raf-1 levels.

17-AG destabilizes mutant p53

Normal p53 has a short half-life and is rapidly degraded by the proteasome. In contrast, mutant p53 is resistant to proteasome degradation which leads to a long half-life and high levels of the mutant protein [37]. SKBR3 cells, which harbor a p53 mutation [11], were treated with increasing doses of 17-AG, and p53 levels were



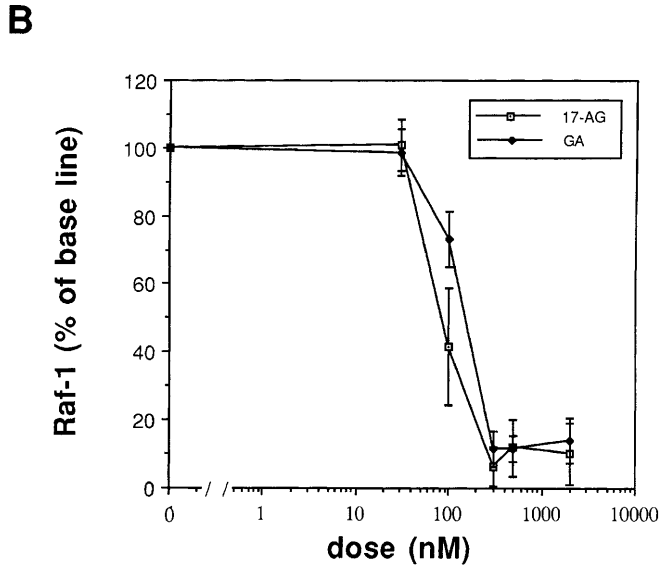


Fig. 4A,B 17-AG destabilizes Raf-1. SKBR3 cells were treated with 17-AG or GA for 16 h at increasing doses. Raf-1 levels were analyzed by Western blotting. **A** Western blot of one of three experiments; **B** results of densitometry of three experiments

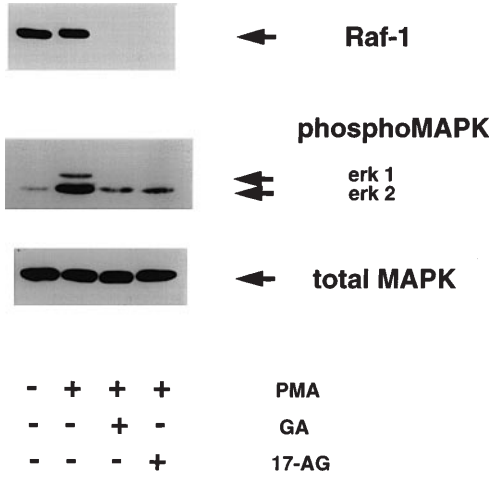
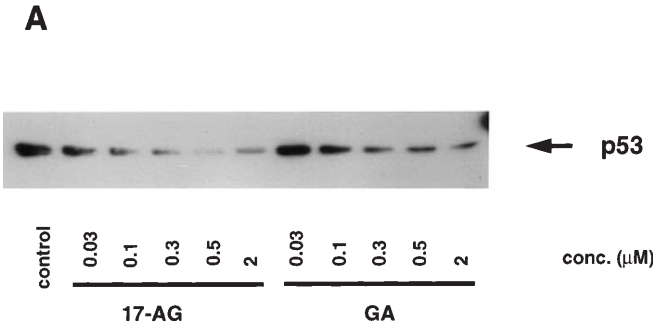


Fig. 5 17-AG blocks the Raf-1-MEK-MAPK signal transduction pathway. NIH3T3 cells were pretreated with or without 17-AG (2 μ*M*) or GA (2 μ*M*) for 16 h, and stimulated with PMA (100 n*M*) for 10 min. Activation of the MAPK signaling cascade was assayed by Western blotting with a phospho-MAPK specific antibody. Western blots of Raf-1 and total MAPK are shown for comparison



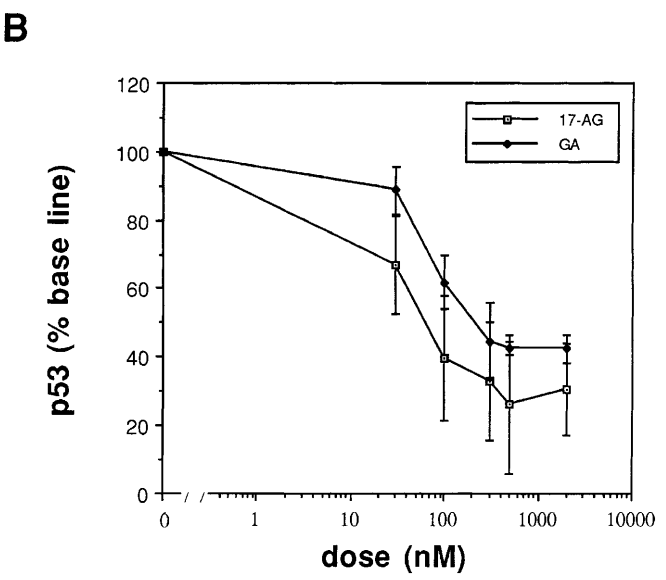


Fig. 6A,B 17-AG destabilizes mutant p53. SKBR3 cells were treated with increasing doses of 17-AG or GA for 6 h. p53 protein levels were assayed by Western blotting. **A** Western blot of one of three experiments; **B** results of densitometry of three experiments

determined after 6 h of treatment by immunoblotting. Mutant p53 levels fell rapidly upon drug treatment and EC_{50} values of 62 n*M* for 17-AG and 210 n*M* for GA treatment were observed (Fig. 6).

17-AG has a similar antiproliferative activity to GA

The antiproliferative activities of 17-AG and its parent compound GA were compared in a standard MTT assay. The IC_{50} values of 17-AG were 4.1 n*M* in SKBR3 cells (Fig. 7A) and 5.2 n*M* in MCF7 cells (Fig. 7B) for 4 days of treatment. GA in comparison showed IC_{50} values of 4.1 n*M* in SKBR3 cells and 10.6 n*M* in MCF7 cells.

Discussion

Targeted drug therapy is one of the most important approaches in the search for new and more effective

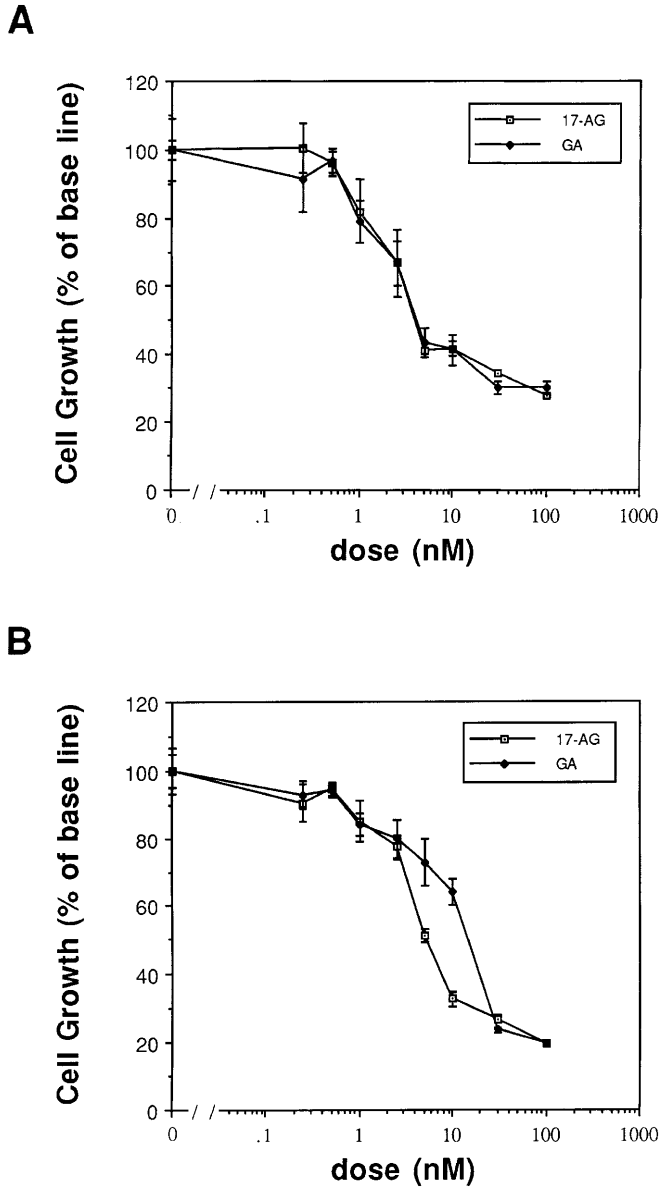


Fig. 7A,B 17-AG has an antiproliferative effect that is similar to GA. SKBR3 cells (**A**) or MCF7 cells (**B**) were plated in 96-well plates and treated with increasing doses of GA or 17-AG. After 4 days, cell growth was measured using an MTT assay and compared with that of untreated cells

cancer treatments. Benzoquinone ansamycins are the first class of drugs described to target HSP90 and its homolog GRP94 and to disrupt the function of these chaperone proteins [28, 36]. The antitumor effects of GA and other ansamycins likely result from the effects on four classes of signaling proteins that depend on chaperone action: (1) receptor and nonreceptor protein kinases, such as $p185^{erbB2}$ [7, 14, 15], EGF-R [17, 18] and v-src [34, 36], (2) serine/threonine kinases such as Raf-1 [23] and CDK4 [27], (3) steroid hormone receptor proteins, including the androgen and estrogen receptor [25, 35] and (4) proteins that regulate the cell cycle and apoptosis, including mutant p53 [3, 4].

Schnur et al. have investigated a significant number of GA derivatives and their structure-activity relationships [22]. They have demonstrated that adding an amino group to the 17 position on the benzoquinone ring can increase biologic activity and stability, while other changes to the molecule, such as introducing bulky or other functional groups in the 17 position destroy biologic activity.

In an endeavor to develop ansamycins as clinical tools, we have evaluated the biologic properties of 17-AG. The compound has been found to be less toxic in vivo than its parent compound GA [21]. High tissue concentrations have been demonstrated in rats and mice [9]. It was the aim of the present investigation to determine whether 17-AG has biologic activity similar to that of GA. First, we sought to determine whether 17-AG binds to HSP90, which has been shown for GA [26, 36]. We used a competition assay with GA-affinity beads and found that 17-AG competed with GA, but with a lower apparent affinity than the parent compound.

Next, we assayed the effects of 17-AG on $p185^{erbB2}$, which is believed to be of special importance in breast cancer. We found that 17-AG was similar to GA in its ability to deplete SKBR3 cells of $p185^{erbB2}$. This effect has been shown to result from GA binding to GRP94, not to $p185^{erbB2}$ [7].

Even though benzoquinone ansamycins were first described as specific tyrosine kinase inhibitors, effects on some serine/threonine kinases have recently been reported [23, 27]. We tested for degradation of Raf-1 because of its importance in mediating growth factor signals to the nucleus [13, 16]. We found that 17-AG treatment destabilized Raf-1 at similar dose levels to GA and blocked the Raf-1-MEK-MAPK signaling cascade.

Because of the special importance of mutant p53, which is found in as many as 50% of cancer cases [2, 12], we assessed the effects of 17-AG on this protein. We found that 17-AG enhanced the degradation of mutant p53. Again, 17-AG demonstrated biologic activity previously described for GA [3]. Finally, 17-AG displayed antiproliferative activity similar to that of GA.

It is not completely clear at this point why we observed in vitro a lower apparent affinity of 17-AG for HSP90 binding compared with GA, while in vivo both drugs achieved biologic effects at comparable concentrations. Differences in drug metabolism might account for these findings, and the various active and inactive metabolites of both GA and 17-AG should be studied in the future.

Taken together, these results demonstrate that 17-AG targets HSP90 and closely resembles GA in mediating several important biologic effects. 17-AG depletes tumor cells of the receptor tyrosine kinase $p185^{erbB2}$. It also depletes cells of the serine-threonine kinase Raf-1 and interrupts signaling through the Raf-1-MEK-MAPK pathway. 17-AG also leads to instability of mutant p53. Importantly, we found 17-AG to be active at concentrations similar to those at which GA is active. Since 17-AG has a profile of biologic actions similar to that of

GA, has antitumor activity and is less toxic than GA based upon animal data [21], we believe 17-AG to be the best benzoquinone ansamycin candidate for a clinical trial.

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