Potent Activity of a Novel Dimeric Heat Shock Protein 90 Inhibitor against Head and Neck Squamous Cell Carcinoma

**In vitro** and **In vivo**

Xiaoying Yin,1 Hong Zhang,2 Francis Burrows,2 Lin Zhang,2 and Carol G. Shores1

**Abstract**

Heat shock protein 90 (Hsp90) is a molecular chaperone that promotes the conformational maturation of numerous client proteins, many of which play critical roles in tumor cell growth and survival. The ansamycin-based Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) is currently in phase I/II clinical testing. However, 17-AAG is difficult to formulate and displays weak activity against some tumors. A novel dimeric ansamycin, EC5, was evaluated for antitumor activity in eight head and neck squamous cell carcinoma (HNSCC) cell lines. Both 17-AAG and EC5 inhibited tumor cell proliferation effectively, but EC5 was more potent, with IC50 below 200 nmol/L in most cell lines tested, including several lines that were resistant to 17-AAG. The inability of 17-AAG to kill JHU12 cells was linked to a defect in retinoblastoma signaling and could be rescued by ectopic expression of p16INK4a. EC5 induced G1 growth arrest of tumor cells and apoptosis, with the degradation of client proteins including epidermal growth factor receptor, c-Raf-1, Akt, and Cdk4 and inhibition of Akt phosphorylation. In vivo, EC5 dramatically reduced the growth rate of established HNSCC xenografts in nude mice and decreased expression of epidermal growth factor receptor and Akt within the xenografts. These results suggest that this novel ansamycin-based Hsp90 inhibitor affects multiple pathways involved in tumor development and progression and may represent a new strategy for the treatment of HNSCC patients.

Head and neck squamous cell carcinoma (HNSCC) is the fifth most common cancer worldwide, with 780,000 new patients diagnosed each year (1) and over 41,000 new cases predicted in United States in 2004 (2). The primary treatment for stage III and IV diseases is either surgery followed by radiation with or without chemotherapy or concurrent radiation and chemotherapy (3–5). Current drug regimens are frequently quite toxic, and despite an increase in organ preservation rates, overall 5-year survival of stage III and IV disease remains less than 50%. The identification of less toxic and more effective agents will have great therapeutic implications for HNSCC patients.

Heat shock protein 90 (Hsp90) is a molecular chaperone that promotes the conformational maturation of “client” proteins and protects them from degradation (6). Many of its known clients are protein kinases or transcription factors involved in multiple signal transduction pathways, including tyrosine kinases [Bcr-Abl, epidermal growth factor receptor (EGFR) family members, c-Met, IGF-R, pp60src], serine/threonine kinases (Akt, Cdk4, β/δ kinases α and β, Raf-1), transcription factors (steroid hormone receptors, p53, Stat3), Mdm2, and telomerase (7–9). Ansamycin treatment also leads to a rapid decline in cyclin D1 levels without affecting cyclin D1 half-life via down-regulation of an Akt-dependent pathway required for cyclin D1 expression (10, 11). Hsp90 is constitutively expressed at 2- to 10-fold higher levels in tumor cells compared with normal cells (12) and the Hsp90 found in tumor cells exists in a functionally distinct molecular form with a 100-fold higher affinity for ansamycin-based inhibitors than Hsp90 derived from normal cells (13). These data show that Hsp90 is a unique therapeutic target for tumor cells, affecting multiple signal pathways (14–16). Targeting multiple pathways may have great utility in advanced cancers, which typically harbor oncogenic changes in multiple signal transduction pathways.

Ansamycin antibiotics are natural products that specifically bind to a conserved pocket in the amino-terminal domain of Hsp90 (17, 18), inhibiting ATP binding and ATP-dependent Hsp90 chaperone activity, leading to the degradation of client proteins (19). 17-Allylamino-17-demethoxygeldanamycin (17-AAG), a geldanamycin-derived Hsp90 inhibitor, has entered clinical trials in cancer patients and shows evidence of biological and clinical activity (20, 21).

17-AAG has some limitations as a therapeutic drug, such as poor solubility and a cumbersome formulation (22). Although it kills tumor cells with high potency in vitro, 17-AAG often only has modest effects on the same tumor cells in vivo. EC5 is a novel ansamycin-based compound, a dimer of geldanamycin,
designed to engage both amino-terminal binding sites on the Hsp90 dimer simultaneously, thus stabilizing the drug-target interaction. EC5 and related ansamyacin dimers have been found to be more potent than 17-AAG after brief drug exposure in vitro and to kill a range of 17-AAG–resistant tumor lines (23). Here we report the potent in vitro and in vivo activities of EC5 against HNSCC cell lines.

Materials and Methods

Reagents. 17-AAG was synthesized from the ansamyacin antibiotic geldanamycin as previously described (24). EC5 was also synthesized from geldanamycin by the following scheme: 3,3'-diamino-N-methylpyrrolidoneamine (13.2 mg, 0.091 mmol) was added dropwise to a flame-dried flask under N2 and stirred at room temperature. The yieldipropylamine (13.2 mg, 0.091 mmol) was added dropwise to a flame-dried flask under N2 and stirred at room temperature. The reaction mixture was diluted with water after 4 hours. A precipitate was formed and filtered to give the crude product. The crude product was chromatographed on silica gel (5% CH3OH/CH2Cl2) to yield EC5 as a purple solid (94.9 mg, 0.079 mmol). Yield: 88.8%.1H nuclear magnetic resonance (CDCl3) δ = 0.95 (d, J = 7 Hz, 6H, 2CH3), 1.00 (d, J = 7 Hz, 6H, 2CH3), 1.69 (m, 4H, 2CH2), 1.74 (m, 4H, 2CH2), 1.76 (s, 6H, 2CH3), 1.83 (m, 2H, 2CH), 2.00 (s, 6H, 2CH3), 2.30 (s, 3H, N-CH3), 2.36 (dd, J = 14Hz, 2H, 2CH), 2.50 (m, 4H, 2CH2), 2.63 (d, 2H, 2CH), 2.75 (m, 2H, 2CH), 3.25 (s, 6H, 2OCH3), 3.35 (s, 6H, 2OCH3), 3.40 (m, 2H, 2CH), 3.50 (m, 4H, 2CH2), 3.68 (m, 2H, 2CH), 4.20 (Bs, 4H, 2NH2), 4.30 (d, J = 10 Hz, 2H, 2CH), 4.80 (Bs, 4H, 2NH2), 5.19 (s, 2H, 2CH), 5.82 (t, J = 15 Hz, 2H, 2CH= =), 5.89 (d, J = 10 Hz, 2H, 2CH= =), 6.59 (t, J = 15 Hz, 2H, 2CH= =). The structures of 17-AAG and EC5 are shown in Fig. 1A and B, respectively. The synthetic route to EC5 is shown in Fig. 1C. The in vitro and to kill a range of 17-AAG–resistant tumor lines (23). Here we report the potent in vitro and in vivo activities of EC5 against HNSCC cell lines.

Materials and Methods

Reagents. 17-AAG was synthesized from the ansamyacin antibiotic geldanamycin as previously described (24). EC5 was also synthesized from geldanamycin by the following scheme: 3,3'-diamino-N-methylpyrrolidoneamine (13.2 mg, 0.091 mmol) was added dropwise to a flame-dried flask under N2 and stirred at room temperature. The yieldipropylamine (13.2 mg, 0.091 mmol) was added dropwise to a flame-dried flask under N2 and stirred at room temperature. The reaction mixture was diluted with water after 4 hours. A precipitate was formed and filtered to give the crude product. The crude product was chromatographed on silica gel (5% CH3OH/CH2Cl2) to yield EC5 as a purple solid (94.9 mg, 0.079 mmol). Yield: 88.8%.1H nuclear magnetic resonance (CDCl3) δ = 0.95 (d, J = 7 Hz, 6H, 2CH3), 1.00 (d, J = 7 Hz, 6H, 2CH3), 1.69 (m, 4H, 2CH2), 1.74 (m, 4H, 2CH2), 1.76 (s, 6H, 2CH3), 1.83 (m, 2H, 2CH), 2.00 (s, 6H, 2CH3), 2.30 (s, 3H, N-CH3), 2.36 (dd, J = 14Hz, 2H, 2CH), 2.50 (m, 4H, 2CH2), 2.63 (d, 2H, 2CH), 2.75 (m, 2H, 2CH), 3.25 (s, 6H, 2OCH3), 3.35 (s, 6H, 2OCH3), 3.40 (m, 2H, 2CH), 3.50 (m, 4H, 2CH2), 3.68 (m, 2H, 2CH), 4.20 (Bs, 4H, 2NH2), 4.30 (d, J = 10 Hz, 2H, 2CH), 4.80 (Bs, 4H, 2NH2), 5.19 (s, 2H, 2CH), 5.82 (t, J = 15 Hz, 2H, 2CH= =), 5.89 (d, J = 10 Hz, 2H, 2CH= =), 6.59 (t, J = 15 Hz, 2H, 2CH= =). The structures of 17-AAG and EC5 are shown in Fig. 1A and B, respectively. The synthetic route to EC5 is shown in Fig. 1C. The compounds were stored as 10 mmol/L stock solutions in DMSO at −20°C, and diluted in medium for use such that the final DMSO concentration did not exceed 0.05%.

Cell culture and cell growth inhibition. Eight HNSCC cell lines were used for this study. UM1, UM6, UM11B, UM14A, and UM35 (kindly provided by Dr. Gregory Wolf, University of Michigan, Ann Arbor, MI) and Cal27 (purchased from American Type Culture Collection, Rockville, MD) were cultured in DMEM supplemented with 10% fetal bovine serum and streptomycin, and 1% nonessential amino acids. JHU12 and JHU12 (provided by Dr. David Sidransky, Johns Hopkins University, Baltimore, MD) were grown in RPMI 1640 with 10% fetal bovine serum and 100 units/mL penicillin G and streptomycin. All cells were cultured in a humidified atmosphere of 5% CO2 at 37°C.

Logarithmically growing cells were counted and plated at 3,000 or 4,000 (for slower growing cell lines) cells per well in triplicates in 96-well plates and incubated overnight. The next morning, 17-AAG or EC5 was added to the cell culture at increasing concentrations (0, 65, 125, 500 nmol/L, 1 μmol/L, and 2 μmol/L). Cell growth inhibition was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Roche Diagnostics, Indianapolis, IN). Briefly, at the 5th day of the culture, 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide labeling reagent was added to each well. After 4-hour incubation, 100 μL of solubilization buffer were added, and the plates were incubated at 37°C overnight. The absorbance of each well was measured at 595 nm using a Vmax kinetics microplate reader. 17-AAG or EC5 concentrations yielding 50% growth inhibition (IC50) were compared with control for each cell line and were expressed as mean values of at least three independent experiments.

Transfection of JHU12 cells. JHU12 cells were plated at 3.5 × 105 cells/35-mm plate. PcDNA3-HA2 vector or PcDNA3-HA2-p16 (kindly provided by Dr. Yue Xiong, University of North Carolina, Chapel Hill, NC) was transfected into the cell with FuGene 6 Transfection Reagent (Roche Diagnostics, GmbH Mannheim, German). Briefly, FuGene 6 and DNA were mixed in serum-free medium in 3 (μL) to 1 (μg) ratio to a total volume of 100 μL. Using green fluorescent protein as a marker, transfection efficiency for the FuGene 6 Transfection Reagent has been about 30% in numerous cell lines tested in our lab. The mixture was incubated for 30 minutes at room temperature, then applied to cells. After 24 hours, 2 μmol/L 17-AAG was added to cells. The cells were incubated for another 48 hours, then all supernatant and cells were collected. An average of 200 cells was counted for each sample, and nonviable cells were identified using trypan blue staining.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay. HNSCC cells were plated in 12-well plates at 30,000 cells per well; 1 μmol/L EC5 was added to each well 24 hours later and incubated for 24 or 48 hours. Cells undergoing apoptosis were detected using ApoTag plus Fluorescein In situ apoptosis detection kit (Inter- gen, Purchase, NY). The cells were fixed with 3% paraformaldehyde for 10 minutes and permeabilized with 0.2% Triton X-100 for 20 minutes at room temperature. Equilibration buffer was applied to each well and incubated for 5 minutes. Working strength terminal deoxynucleotidyl transferase enzyme (reaction buffer/terminal deoxynucleotidyl
transferrase enzyme, 7:3) was added, followed by incubation for 1 hour at 37°C in a humidified chamber. The reaction was then stopped by adding prewarmed stop/wash buffer. The apoptotic cells were labeled with anti-digoxigenin fluorescein and visualized under a fluorescent microscope.

**Cell cycle analysis.** Cell cycle distribution was measured before and after HNSCC cells exposure to 1 µmol/L EC5. Cells were collected at 24 and 48 hours after exposure to EC5, fixed with 70% ethanol, incubated with propidium iodide (20 µg/mL) and RNase (200 µg/mL) for 30 minutes at 37°C, and analyzed by flow cytometer (FACS, Becton Dickinson, Franklin Lakes, NJ). ModFit II software program was used for cell cycle distribution analysis.

**Protein extraction and Western blotting.** Logarithmically growing HNSCC cells were harvested before and after 24- or 48-hour exposure to 1 µmol/L EC5. Protein extracts were prepared by lysis cells in NP40 lysis buffer [50 mmol/L Tris-HCl, 150 mmol/L NaCl, 50 mmol/L NaF, 0.5% NP40, and 1 table of protease inhibitors cocktail (Boehringer Mannheim, Germany) per 100 mL]. The protein extracts were quantitated using the Bradford protein assay (Bio-Rad, Hercules, CA).

For Western blotting, 40 µg of protein were electrophoresed through 12% SDS polyacrylamide gels under denaturing conditions and transferred to nitrocellulose membranes. The membranes were blocked in PBS, 0.1% Tween 20, and 5% nonfat dry milk, then incubated with primary antibodies (0.8 µg/mL anti-c-Raf-1 monoclonal antibody, 0.8 µg/mL anti-Akt monoclonal antibody, or 0.8 µg/mL anti–cyclin D1 monoclonal antibody; Santa Cruz, Santa Cruz, CA), 0.4 µg/mL anti-EGFR rabbit polyclonal antibody (kindly provided by Dr. Sholton Earp, University of North Carolina), 0.2 µg/mL anti–phospho-Akt polyclonal antibody (Cell Signaling, Beverly, MA), and 0.2 µg/mL antitubulin monoclonal antibody (Roche Molecular Biochemicals, Indianapolis, IN), washed, then incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit second antibody (Santa Cruz, and Amersham Biotics, Piscataway, NJ). Specific antigen-antibody interaction was detected with enhanced chemiluminescence Western blotting detection reagents (Amersham Biotics). EC5 at 1 µmol/L was used for all biochemical assays as this is the concentration that can induce complete inhibition of Hsp90.

**Animal studies.** Experiments were conducted in accordance to the USPHS Policy on Humane Care and Use of Laboratory Animals and the rules and regulations of the University of North Carolina Institutional Animal Care and Use Committee. Four- to five-week-old nude mice were obtained from Charles River Laboratory (Wilmington, MA) and maintained in ventilated caging. JHU122 cells were harvested, washed with 1× PBS, and suspended in PBS at 3 × 10^7/100 mL, and then inoculated s.c. into the flank of mice. After tumor achieved a size of 2 mm in diameter (7-10 days), EC5 emulsion or placebo emulsion was i.p. injected into the mice at 50 mg/kg/d, 3 days/wk (Monday, Wednesday, and Friday) for 4 weeks, or 30 mg/kg/d, 5 days/wk (Monday through Friday) for 4 weeks in groups of five mice each. EC5 and 17-AAG were formulated in emulsion containing Phospholipon 90 G, Miglyol 812 N, sucrose, and water. The tumor size and mice weight were measured weekly. At the time of sacrifice, tumors were lysed in 100% formalin for histology to confirm tumor structure.

**Results**

**Antiproliferative effects of 17-AAG and EC5 on head and neck squamous cell carcinoma.** The eight human HNSCC cell lines were chosen for this study because they (a) all express high level of EGFR, (b) have a wide range of sensitivity to the commonly used chemotherapy agent cisplatin, and (c) have known p53 and retinoblastoma status (25). The cell lines were treated with increasing concentrations of 17-AAG or EC5 for 5 days and evaluated for effects on cell viability. Both 17-AAG and EC5 effectively inhibited HNSCC cell proliferation, but EC5 was generally more potent, with IC_{50} values below 200 nmol/L in six of eight cell lines (Fig. 2A). The mean IC_{50} for EC5 was 215 ± 26 nmol/L, as opposed to 500 ± 216 nmol/L for 17-AAG. JHU12, JHU22, UM1, and UM6 express wild-type p53 and vary in sensitivity to both compounds, demonstrating no clear effect of p53 status on HNSCC sensitivity to either drug. In all cell lines, EC5 and 17-AAG induced growth inhibition in a dose-dependent manner. Representative growth inhibition curves for EC5 are shown in Fig. 2B.

**17-AAG sensitivity is retinoblastoma pathway dependent in JHU12 cells.** One cell line, JHU12, was particularly resistant to 17-AAG but retained sensitivity to EC5. Cells with defects in retinoblastoma signaling have been reported to be insensitive to 17-AAG but not to other Hsp90 inhibitors (26). The mechanism of this differential sensitivity is unknown. Although all HNSCC lines used here express retinoblastoma, JHU12 has a methylated promoter in the gene for p16^INK4a, an upstream regulator of

---

3 X. Yin, unpublished data.
retinoblastoma, which results in decreased retinoblastoma activity (27). To examine if restoration of the retinoblastoma pathway can bestow 17-AAG sensitivity on these cells, p16\textsuperscript{INK4a} was transiently transfected into JHU12 and the cells were exposed to 17-AAG. Compared to vector-transfected cells, the sensitivity to 17-AAG increased by about 30% (Fig. 3), indicating that the sensitivity is retinoblastoma dependent.

**EC5 inhibits head and neck squamous cell carcinoma cell growth by causing p53-independent G\textsubscript{1} growth arrest and apoptosis.** HNSCC cell lines with either wild-type or mutant p53 arrested in G\textsubscript{1} phase after 24-hour exposure to 1 \textmu{}mol/L EC5, a dose that induces complete growth arrest. Using flow cytometry, the G\textsubscript{1}-G\textsubscript{0} population increased by 20% to 35% and S phase decreased by 65% to 80% in all cell lines examined. Two representative flow cytometry data plots of HNSCC cell lines are shown in Fig. 4. To investigate whether EC5 induced apoptosis in HNSCC cells, cells grown in 12-well plates were treated with 1 \textmu{}mol/L EC5 and assayed for the presence of apoptotic cells with an in situ apoptosis detection kit and visualized under the fluorescence microscope. As shown in Fig. 5, apoptosis could easily be detected in JHU22 cells after 24-hour treatment with EC5. By 48 hours, massive apoptosis was seen, whereas the untreated cultures had almost undetectable levels of apoptotic cells.

**EC5 induces down-regulation of heat shock protein 90 client proteins.** Figure 6A and B illustrates the effect of EC5 treatment on Hsp90 client protein expression in HNSCC cell lines. Protein lysate was collected before and after 24 or 48 hours of exposure of HNSCC cells to 1 \textmu{}mol/L EC5, and subjected to immunoblot analysis. EGFR, Akt, phospho-Akt, c-Raf-1, and cyclin D1 were expressed in all the cell lines. A significant decrease of EGFR protein expression was detected at 24 hours after EC5 treatment in most cell lines, but in the UM11B and UM35 cell lines only a slight decrease was detected after 48 hours. Both Akt kinase and phospho-Akt expressions were significantly reduced after 24 hours in most cell lines. Whereas the expression of phospho-Akt was almost undetectable in all the cell lines at 48 hours (24 hours in JHU22, JHU12, and UM14A cells), the reduction of Akt expression level in UM1 and UM11B was minimal and only seen after 48 hours of EC5 treatment. c-Raf-1 expression was decreased in all the cell lines 24 hours after EC5 exposure and more dramatically at 48 hours. In the four p53 wild-type cell lines (JHU12, JHU22, UM1, and UM6), c-Raf-1 was almost undetectable after 48 hours of EC5 treatment. The duration of suppression of cyclin D expression varied among the cell lines: 24 hours after exposure to EC5, cyclin D1 protein expression was decreased in all cell lines tested whereas at 48 hours, levels had returned to baseline in some cell lines.

**EC5 inhibits tumor growth in nude mice accompanied by reduction in expression of heat shock protein 90 client proteins.** We examined the effects of i.p. injection of EC5 on the growth of established JHU22 tumors in the flanks of nude mice. Two dosing schedules were employed: (a) continuous schedule of EC5 30 mg/kg injected through Friday for 4 weeks, and (b) intermittent schedule of 50 mg/kg/d Monday, Wednesday, and Friday. Both schedules caused inhibition of tumor growth, with the intermittent schedule inhibiting tumor growth more effectively than the continuous schedule (83% versus 68%). Weight loss in EC5 treatment groups was less than 5% relative to controls, suggesting that the drug was well tolerated (data not shown). The mean tumor volume in EC5-treated groups versus the control group was significantly reduced at 4 weeks (Fig. 7A) and the drug also markedly reduced the expression of EGFR, Akt, and Raf-1 in JHU22 xenografts (Fig. 7B). These data show that EC5 inhibits client protein expression and the growth of HNSCC cells at tolerable doses in vivo.

**Discussion**

In this report, we describe the effects of EC5, a novel ansamycin-based Hsp90 inhibitor, on HNSCC tumor growth using a panel of eight HNSCC cell lines and a xenograft model. Like other Hsp90 inhibitors, EC5 inhibits the molecular chaperone function of Hsp90, an activity that is crucial for maintaining stability and function of numerous key oncogenic signaling molecules such as c-Raf-1 and several members of the EGFR family of receptor tyrosine kinases (28–30). EC5 is a dimeric Hsp90 inhibitor, designed and optimized to bind and inhibit the chaperoning activity of Hsp90, which is also a dimer in cells. The current study in human HNSCC documents that EC5 effectively inhibits tumor cell growth in vitro, concomitant with induction of cell cycle arrest, apoptosis, and down-regulation of oncprotein expression, and inhibits tumor cell growth in nude mice.

ATP binding and hydrolysis cause conformational changes in HSP90 that are required for its function (17, 18). Ansamycin-based compounds such as geldanamycin, 17-AAG, and EC5 prevent the formation of the “mature” p23 multichaperone complex by competing with ATP for binding to Hsp90, leading ultimately, by an incompletely understood mechanism, to destabilization and proteasomal degradation of client proteins (19, 29). The time course of client protein loss is variable. Rapidly degraded clients, such as Raf-1, probably interact continuously with Hsp90 and require binding for stability
Clients that degrade with a slower time course, such as Akt, probably interact only transiently with the chaperone during maturation and deplete at rates determined by the protein half-life.

Inhibition of Hsp90 by benzoquinone ansamycin antibiotics induces antiproliferative effects in many human cancer cell lines in vitro with variable sensitivity (32–34). To our knowledge, the effect of this group of antitumor agents in HNSCC has not been reported. In this study, 17-AAG and EC5 induced antiproliferative effects in all the HNSCC cell lines. The IC50 after 5 days of exposure to EC5 was lower than 17-AAG in most cell lines examined. Preliminary work has indicated that dimeric ansamycins of the class represented here by EC5 have an extended duration of action in cells and induce apoptosis more efficiently than 17-AAG in some malignant cell types (24).

The JHU12 cell line was particularly resistant to 17-AAG but retained sensitivity to EC5. In this cell line, the p16INK4a promoter is methylated, leading to decreased expression of this cyclin kinase inhibitor and constitutive activation of cyclin dependent kinases (27). Subsequently, retinoblastoma is phosphorylated and E2F released, effectively rendering the cells retinoblastoma negative and without effective G1 checkpoint control. In HNSCC, deletion and loss of expression of retinoblastoma is rare and phosphorylation of the p16INK4a promoter is relatively common (35). Transient restoration of p16INK4a expression increased the sensitivity of JHU12 cells to 17-AAG. Srethapakdi et al. (11) reported that treatment with the 17-AAG parent compound geldanamycin induced G1 arrest in cell lines with a functional retinoblastoma gene product, but not in those lacking functional retinoblastoma. These investigators suggest that the differentiation is cyclin D dependent, with retinoblastoma-negative cells being independent of geldanamycin-mediated cyclin D loss. In our study, restoring effective control of retinoblastoma may render the cells once again sensitive to decreasing cyclin D protein levels.

To determine whether growth inhibition by EC5 is attributable to inhibition of cell proliferation alone or a combination of growth arrest and induction of cell death, we assessed whether EC5 induces apoptosis by terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling analysis. Our results show a time-dependent increase in apoptotic cell population in HNSCC cells after EC5 treatment. It is reported that 17-AAG induces apoptosis and this was shown by morphologic changes and confirmed by proteolytic cleavage of poly(ADP-ribose) polymerase and caspase 3 (36), and induction of G1 arrest in HNSCC cells commonly leads to apoptotic cell death (37). Taken together, these suggest that the antiproliferative effect by EC5 in HNSCC can be explained by G1 arrest followed by apoptotic cell death.

The antitumor effects of ansamycins are ultimately due to inhibition of the expression and activity of oncoproteins, such as EGFR and p185erbB2, and inhibition of signaling pathways...
such as phosphatidylinositol 3-kinase/Akt and Raf/mitogen-activated protein/extracellular signal-regulated kinase kinase. HNSCC is of particular interest with respect to 17-AAG and EC5 because of the high rate of EGFR overexpression (38), the activation of Ras-Raf-mitogen-activated protein/extracellular signal-regulated kinase kinase-mitogen-activated protein kinase cascade (39), and sensitivity to specific anti-EGFR antibodies (40) and tyrosine kinase-specific inhibitors (41, 42). Downregulation of EGFR expression by EC5 was readily detected in six cell lines. In two cell lines, only minimal EGFR reduction was detected after 48 hours of EC5 treatment, although these two lines are sensitive to EC5-induced growth inhibition, suggesting that other pathways may be involved. Ansamycins inhibit EGFR signaling by both accelerated degradation and intracellular retention of the EGFR (43). Raf-1 plays an important role in the regulation of proliferation, differentiation, and apoptosis (44), and is an attractive target for therapy. Raf-1 inhibition by antisense nucleotide treatment induced potent antiproliferative effects in tumor cell lines (45), and depletion of Raf-1 by treatment with ansamycins induces growth inhibition and apoptosis of tumor cells (46, 47). We confirmed the down-regulation of c-Raf-1 with EC5 treatment and antitumor activity in HNSCC.

Akt is critical in a number of cell survival pathways, including the phosphatidylinositol 3-kinase pathway and signaling through nuclear factor \( \kappa \)B (48, 49). It positively regulates cell growth and inhibits induction of programmed cell death (50). Treatment of tumor cells with 17-AAG leads to degradation of Akt and a rapid loss of Akt activity in breast and prostate cancer (51, 34). We also showed that Akt levels declined in most...
HNSCC cell lines and Akt phosphorylation was significantly inhibited in all cell lines by EC5 treatment. The two cell lines that had minimal reductions in EGFR levels also exhibited less pronounced Akt degradation after EC5 treatment. However, Akt phosphorylation was markedly reduced, suggesting that Akt is targeted at several levels by Hsp90 inhibition. Similarly, Basso et al. (51) reported that 17-AAG treatment of breast cancer cells led to a rapid decrease in AKT activity that preceded loss of the protein and was attributed to early inhibition of upstream signaling via the HER-2/HER-3 axis. Consistent with our in vitro study of EC5 in HNSCC cell lines, we found that EC5 treatment also inhibited xenograft tumor growth and reduced the expression of Akt, EGFR, and Raf-1 in vivo.

EC5 was more effective against HNSCC lines than 17-AAG. The reasons for this are still under investigation, but are probably related to the unique structure of the EC5 series of compounds (23, 24). EC5 is one of the most active of a series of divalent Hsp90 inhibitors and the length and flexibility of the linker between the two geldanamycin pharmacophores is critical to the potency of EC5 and other highly active dimers. Hsp90 is an obligate dimer and the two amino-terminal ATP binding sites are brought into close apposition when the protein is activated (52), suggesting that EC5 may engage both sites simultaneously. This binding might be effectively irreversible, and dimeric Hsp90 inhibitors have been found to have an extended duration of action in cells (23). Thus, under conditions of brief exposure, 17-AAG suppressed client protein expression for only 24 hours and induced a transient growth arrest. Under the same conditions, EC5 and related dimers inhibited client expression for >72 hours and caused massive apoptosis in a panel of malignant cells (23, 24). Taken together, these data suggest that EC5 may have improved activity in HNSCC due to a prolonged inhibition of Hsp90 activity.

Most cancers have multiple genetic aberrations and altered signal transduction pathways. Targeting one of these altered pathways usually fails to achieve sustained control of tumor cell growth. Inhibition of Hsp90 promises to be an effective form of cancer chemotherapy because multiple oncoproteins and pathways can be affected at the same time (9), as we and others have shown. 17-AAG is currently being studied in at least 14 open clinical trials, and Conforma Therapeutics has opened two phase I trials of CFN1010, a form of 17-AAG. In conclusion, EC5 inhibits HNSCC growth in vitro and in vivo, down-regulating EGFR, Akt, phospho-Akt, Raf-1, and cyclooxygenase-1 expressions in HNSCC and inducing GI arrest and subsequent apoptotic cell death. Our study suggests that EC5 might be a promising new alternative treatment for HNSCC patients who fail to respond to traditional chemotherapy agents.

References


Clinical Cancer Research

Potent Activity of a Novel Dimeric Heat Shock Protein 90 Inhibitor against Head and Neck Squamous Cell Carcinoma

In vitro and In vivo

Xiaoying Yin, Hong Zhang, Francis Burrows, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/11/10/3889

Cited articles
This article cites 50 articles, 14 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/11/10/3889.full.html#ref-list-1

Citing articles
This article has been cited by 8 HighWire-hosted articles. Access the articles at:
/content/11/10/3889.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.