Inhibition of Hsp90 Down-regulates Mutant Epidermal Growth Factor Receptor (EGFR) Expression and Sensitizes EGFR Mutant Tumors to Paclitaxel

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Abstract

Mutations in the kinase domain of the epidermal growth factor receptor (EGFR) are found in a subset of patients with lung cancer and correlate with response to EGFR tyrosine kinase inhibitors (TKI). Resistance to these agents invariably develops, and current treatment strategies have limited efficacy in this setting. Hsp90 inhibitors, such as 17-allylamino-17-demethoxygeldanamycin (17-AAG), induce the degradation of EGFR and other Hsp90 interacting proteins and may thus have utility in tumors dependent upon sensitive Hsp90 clients. We find that the EGFR mutations found most commonly in patients with lung adenocarcinoma who respond to EGFR TKIs are potently degraded by 17-AAG. Although the expression of wild-type EGFR was also down-regulated by 17-AAG, its degradation required higher concentrations of drug and a longer duration of drug exposure. In animal models, a single dose of 17-AAG was sufficient to induce degradation of mutant EGFR and inhibit downstream signaling. 17-AAG treatment, at its maximal tolerated dose, caused a significant delay in H3255 (L858R EGFR) xenograft growth but was less effective than the EGFR TKI gefitinib. 17-AAG alone delayed, but did not completely inhibit, the growth of H1650 and H1975 xenografts, two EGFR mutant models which show intermediate and high levels of gefitinib resistance. 17-AAG could be safely coadministered with paclitaxel, and the combination was significantly more effective than either drug alone. These data suggest that Hsp90 inhibition in combination with chemotherapy may represent an effective treatment strategy for patients whose tumors express EGFR kinase domain mutations, including those with *de novo* and acquired resistance to EGFR TKIs. [Cancer Res 2008;68(2):589–96]

Introduction

Activating mutations in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) are found in ~10% of non–small cell lung cancers (NSCLC) in the United States and in as many as 25% of tumors from East Asian populations (1–3). The most common EGFR mutations observed in lung cancer are small in-frame deletions in exon 19 and the L858R point mutation in exon 21. These mutations induce oncogenic transformation in both fibroblasts and lung epithelial cells *in vitro* and in transgenic mice through constitutive activation of EGFR (4–6). The exons 19 and 21 mutations also confer sensitivity to EGFR tyrosine kinase inhibitors (TKI), including gefitinib and erlotinib (4). Although initially effective in many patients with NSCLC, resistance to gefitinib and erlotinib invariably develops (7). One mechanism of acquired resistance is selection for a second threonine-to-methionine substitution at position 790 (T790M; ref. 8). This second-site mutation in EGFR, which is analogous to the T315I "gatekeeper" mutation that confers resistance of bcr-abl to imatinib (9), is predicted to block binding of erlotinib and gefitinib to the EGFR ATP-binding domain.

Hsp90 is a protein chaperone with a role in protein folding, stability, and maturation. Hsp90 clients include a subset of kinases, steroid receptors, and transcription factors, many of which are dysregulated in human cancer (10–15). Certain mutated oncoproteins, including bcr-abl and V600E BRAF, are clients of Hsp90, whereas their wild-type counterparts are either not dependent or only weakly dependent upon Hsp90 (16–18). It is hypothesized that these "gain of function" mutations are unable to fold properly in the absence of Hsp90. These oncoproteins, therefore, gain the ability to induce transformation at the expense of greater dependence upon Hsp90 chaperone function.

Our understanding of Hsp90 biology is, in great degree, derived from the study of geldanamycin and radicicol, natural products that bind a regulatory pocket in the N-terminal domain of the protein, which is conserved across species (19–21). The physiologic ligands of the pocket are ATP and ADP. Binding of geldanamycin, radicicol, or their analogues mimics the effects of ADP, resulting in the degradation of proteins that require Hsp90 for maturation or stability (22, 23). Geldanamycin proved too toxic for human use, but several derivatives, including 17-allylamino-17-demethoxy geldanamycin (17-AAG) are currently being tested in patients with promising early results in several cancer types, including HER2 amplified breast cancers, myeloma, and acute myelogenous leukemia (24).

Studies by several groups have shown that wild-type EGFR is relatively insensitive to degradation by inhibitors of Hsp90 compared with HER2 (25, 26). It has been reported that, whereas both the mature and nascent forms of HER2 are degraded by geldanamycin, only nascent EGFR is Hsp90 dependent (26). Recently, we and others have shown that exons 19 and 21 EGFR kinase domain mutants are degraded by Hsp90 inhibitors (27, 28). These data suggest that Hsp90 is permissive for the development of mutant EGFR-dependent lung cancers and, therefore, may be an

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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effective clinical strategy in patients whose tumors express activating mutations of EGFR, including those who have developed clinical resistance to EGFR TKIs.

We now show that 17-AAG, the Hsp90 inhibitor being tested in phases 1 and 2 clinical trials, can induce the degradation of mutant EGFR, but not wild-type EGFR, in xenograft tumors at nontoxic doses. The maximal effect of 17-AAG on mutant EGFR expression was observed 6 h posttreatment, with recovery to baseline by 48 h. 17-AAG was effective in delaying xenograft tumor growth, but was significantly less effective than gefitinib in a gefitinib-sensitive, EGFR mutant–driven model. This may have been in part due to the inability, because of toxicity limitations, to continuously inhibit EGFR signaling with 17-AAG. Although only modest antitumor activity was observed in EGFR mutant models with 17-AAG alone, full doses of 17-AAG and paclitaxel could be given without evidence of additive toxicity, and the combination was significantly more effective than either agent alone. These data suggest that Hsp90 inhibitors may be most effective in combination with cytotoxics in lung adenocarcinoma patients with TKI-resistant EGFR mutations.

Materials and Methods

Chemicals and antibodies. 17-AAG was obtained from Conforma Therapeutics and dissolved in DMSO to yield 50 mg/mL stock solutions and stored at −20°C. The EPL diluent was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute. Paclitaxel was obtained from Bristol-Myers Squibb and stored at room temperature. Gefitinib was obtained from AstraZeneca Pharmaceuticals. For cell culture studies, gefitinib was dissolved in DMSO and stored at −20°C. For animal studies, it was dissolved in distilled water with 0.5% lactic acid (85%; Fisher Scientific) to prepare a stock solution and stored at 4°C. The following antibodies were used: EGFR, phosphorylated EGFR (Y845, Y1068), Akt, phosphorylated Akt, p44/42 mitogen-activated protein kinase (MAPK), phosphorylated MAPK (Cell Signaling), Raf-1, cyclin D1 (Santa Cruz), p85, and HER2 (Upstate Biotechnology).

Cell culture. All cell lines, except for NCI-H3255, were obtained from the American Type Culture Collection. NCI-H3255 cells were provided by Drs. B. Johnson and P. Janne (Dana-Farber Cancer Institute). A431 was maintained in a 1:1 mixture of DME:F-12, A549 in F12K, and NCI-H441, H1650, H1666, H1734, H1975, H3255, Calu-1, and Colo205 in RPMI 1640. All cells were supplemented with 2 mmol/L L-glutamine, 50 units/mL each of penicillin and streptomycin, and 10% fetal bovine serum (Gemini Bioproducts). NIH/3T3 cells expressing wild-type and mutant EGFR were generated as previously reported (4) and were maintained in puromycin (2 μg/mL) and DMEM supplemented with 2 mmol/L L-glutamine, 50 units/mL each of penicillin and streptomycin, and 10% calf serum (Life Technologies/Invitrogen). For cell proliferation/viability studies, cells ($2–4 \times 10^3$ per well) were plated in 96-well plates. After 24 h, cells were treated with multiple concentrations of drug and grown in the presence or absence of drug for 72 h. Cell viability was determined by using the AlamarBlue assay (TREK Diagnostic Systems) according to the manufacturer's instructions, and the plates were read by a fluorescence spectrophotometer. All experiments were repeated at least three times.

Western blotting. Treated cells were harvested, washed with PBS, and lysed in NP40 lysis buffer [50 mmol/L Tris (pH 7.4), 1% NP40, 150 mmol/L NaCl, 40 mmol/L NaF, 1 mmol/L Na_3VO_4, 1 mmol/L phenylmethylsulfonylfluoride, and 10 μg/mL each of leupeptin, aprotinin, and soybean trypsin inhibitor] for 30 min on ice. Cell lysates were centrifuged at 13,200 rpm for 10 min, and the protein concentrations were determined by bicinchoninic acid assay (Pierce Biotechnology). Total protein (50 μg) was resolved by SDS-PAGE and transferred onto nitrocellulose membranes. Blots were probed with antibodies to detect the protein of interests overnight at 4°C.

Animal studies. Four-week-old to six-week-old nu/nu athymic female mice were obtained from National Cancer Institute, Frederick Cancer Center, and maintained in ventilated caging. Experiments were carried out under an Institutional Animal Care and Use Committee–approved protocol, and institutional guidelines for the proper and humane use of animals were followed. Xenografts were generated by injecting 7 to 10×10^6 cells together with Matrigel (BD Biosciences). Before treatment, mice were randomized to receive 17-AAG, gefitinib, paclitaxel, paclitaxel and 17-AAG, or the vehicles only as control. Before injection, 17-AAG was diluted with EPL solution. Paclitaxel was diluted in normal saline at a minimum 1:3 dilution. Both agents were given by i.p. injection. Gefitinib was formulated in distilled water with 0.5% lactic acid and given by oral gavages. Tumor size was measured twice weekly by caliper in control and treatment groups. The average tumor volume in each group was expressed in cubic millimeter and calculated using the formula $\pi / 6 \times (\text{large diameter}) \times (\text{small diameter})^2$. For statistical purposes, the average tumor burden for each mouse was characterized using the area under the volume–time profile and compared across groups using a Wilcoxon test with the exact reference distribution. Mice were euthanized by CO_2. To prepare tumor lysates, tumor tissues was homogenized in SDS lysis buffer [50 mmol/L Tris-HCl (pH 7.4) and 2% SDS].

Results

17-AAG causes down-regulation of mutant forms of EGFR. We and others have previously shown that Hsp90 inhibitors down-regulate the expression of mutant forms of EGFR (27, 28). To directly compare the effect of Hsp90 inhibition on the expression of wild-type and mutant EGFR, we used NIH/3T3 cells stably expressing either wild-type EGFR, L858R mutant EGFR, or EGFR with a L747_E749 exon 19 deletion. An isogenic model was chosen for these studies, as sensitivity of individual Hsp90 clients to 17-AAG–induced degradation varies among cancer cell lines as a result of differences in DT-diaphorase and Pgp expression (29, 30). As shown in Fig. 1, both the L858R and exon 19 deletion EGFR mutants were more sensitive to 17-AAG–induced degradation than wild-type EGFR. Down-regulation of the mutant forms of EGFR required lower concentrations of drug and occurred at markedly earlier time points (3–6 h versus 12–24 h). The Hsp90 client Raf-1 was also notably less sensitive to 17-AAG–induced degradation than mutant forms of EGFR. No difference in the sensitivity of Raf-1 or other Hsp90 clients was observed in the wild-type and mutant EGFR-transfected NIH/3T3 cells (Fig. 1*A* and data not shown).

Similarly, 17-AAG treatment resulted in reduced expression of mutant forms of EGFR in lung adenocarcinoma cell lines (Fig. 2 and Supplementary Fig. S1). In addition to the L858R mutant (found in H3255) and the E746_A750 deletion mutant (found in H1650), the expression of the L858R/T790M double mutant (found in H1975), which is resistant to erlotinib, was also down-regulated in response to 17-AAG treatment. Loss of L858R/T790M EGFR expression in H1975 cells was accompanied by inhibition of MAPK and Akt signaling. The *in vitro* sensitivity of lung cancer cell lines to 17-AAG did not, however, correlate with EGFR mutational status. Lung cancer cell lines with both mutant and wild-type EGFR were sensitive to 17-AAG with IC_{50}s ranging from 3 to 90 nmol/L. These results are not surprising, as numerous proteins require Hsp90 and are effectively degraded by 17-AAG *in vitro*. As an example, at 24 h, both phospho-Akt and phospho-MAPK expression was down-regulated by 17-AAG in Calu-1 cells (wild-type EGFR) despite the minimal effect of 17-AAG on EGFR expression in these cells. Inhibition of these pathways may be mediated by degradation of upstream receptor tyrosine kinases that require Hsp90 for stability or activation and/or by down-regulation of Raf-1 and Akt, both of which are Hsp90 clients (13, 31).

17-AAG down-regulates the expression of mutant EGFR and inhibits the growth of lung adenocarcinoma xenografts with exons 19 and 21 mutations. Hsp90 inhibitors degrade a wide

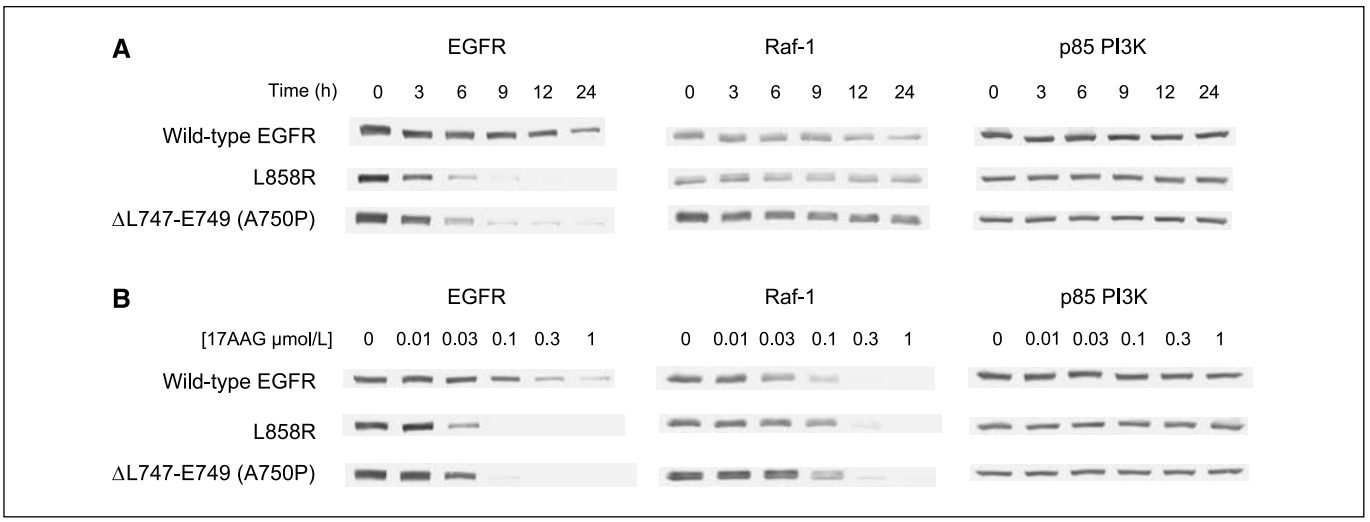

Figure 1. Mutant forms of EGFR are more sensitive than wild-type EGFR to 17-AAG–induced degradation. *A,* NIH-3T3 cells were stably transfected with wild-type and mutant forms of EGFR (L858R and ΔL747_E749, A750P). Immunoblots of EGFR, Raf-1, and p85 phosphatidylinositol 3-kinase (PI3K) show that mutant forms of EGFR were degraded at earlier time points than wild-type EGFR after treatment with 0.1 μmol/L of 17-AAG. p85 PI3K, a protein unaffected by Hsp90 inhibition, was included as a loading control. *B,* immunoblots of EGFR, Raf-1, and p85 PI3K, showing that lower concentrations of 17-AAG were required to down-regulate mutant forms of EGFR (L858R and ΔL747_E749, A750P) compared with wild-type EGFR. Cells were harvested 24 h after treatment with 17-AAG.

spectrum of client proteins in cellular models in tissue culture. Many of these Hsp90 clients, however, are only marginally affected or not affected at all in tumors *in vivo* when 17-AAG is given at its maximally tolerated dose. This may account both for the therapeutic index of the drug and the relatively narrow spectrum of antitumor activity observed in humans in early clinical trials. Thus far, the protein that has been identified as most sensitive to 17-AAG–induced degradation is HER2, and 17-AAG has shown

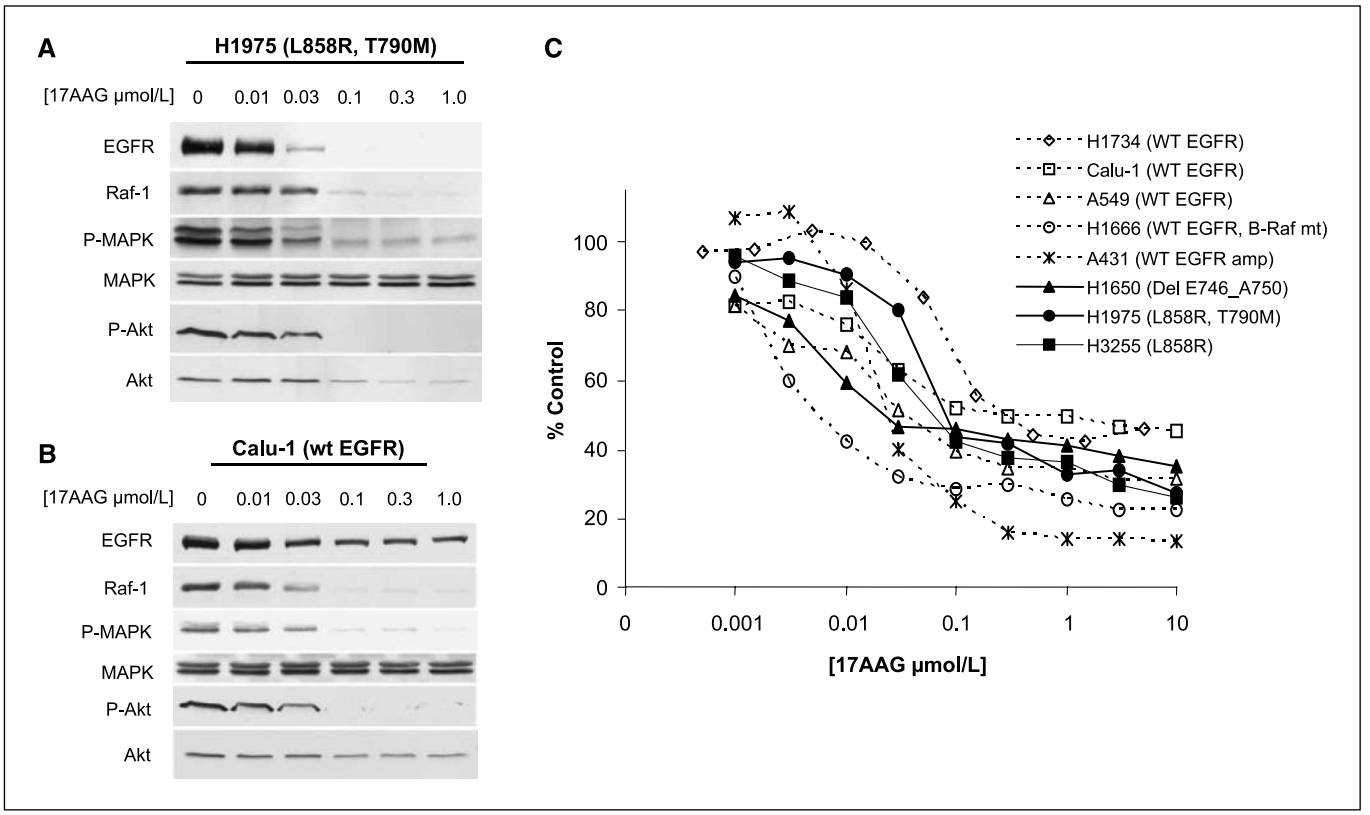

Figure 2. 17-AAG induced the degradation of the L858R, T790M, EGFR mutant in H1975 lung adenocarcinoma cells. *A,* immunoblots showing that 17-AAG causes degradation of mutant EGFR and down-regulation of phospho-MAPK and phospho-Akt expression in H1975 cells. *B,* in Calu-1 cells (wild-type EGFR), EGFR expression is only minimally affected by 17-AAG. However, other Hsp90 clients, such as Raf-1, are degraded in Calu-1. Cells were treated with 17-AAG for 24 h at the doses indicated. *C,* both EGFR mutant and EGFR wild-type lung cancer cell lines were sensitive to 17-AAG *in vitro*. Cellular proliferation was assayed at 72 h by Alamar Blue and graphed as a percentage of DMSO-treated cells.

promising activity in HER2-dependent breast cancer (32). In contrast, mutant BRAF is less sensitive than HER2 to 17-AAG–mediated degradation, and *in vivo* models of BRAF-dependent melanomas are correspondingly less sensitive than HER2-dependent breast tumors to 17-AAG as well (Supplementary Fig. S2; refs. 17, 33).

To examine whether Hsp90 inhibitors were potentially relevant for the treatment of tumors driven by mutant EGFR, we determined the effects of 17-AAG on mutant and wild-type EGFR expression in lung cancer xenografts *in vivo*. Mice bearing H1650 (ΔE746_A750 EGFR), H3255 (L858R EGFR), H1975 (L858R, T790M EGFR), and A549 (wild type EGFR) xenografts were used in these studies. Mice were initially treated by i.p. injection with a single dose of 50 to 100 mg/kg of 17-AAG. Control mice were treated with the EPL diluent vehicle alone. Mice were sacrificed 6 h after drug treatment, and the expression of EGFR was compared in treated and control mice. At 6 h, 17-AAG potently reduced the expression of mutant EGFR (ΔE746_A750) by >95% in H1650 xenografts (Fig. 3). Down-regulation of EGFR was accompanied by inhibition of MAPK signaling and reduced cyclin D1 expression in the tumors. Partial down-regulation of phosphorylated Akt expression was also observed. Similar effects were observed in mice bearing H3255 and H1975 xenografts (Fig. 3*C* and data not shown).

To assess the kinetics of the effect of 17-AAG treatment on mutant EGFR expression and downstream signaling, mice were treated with a single dose of 75 mg/kg of 17-AAG and sacrificed from 1 to 48 h posttreatment. The maximal effect of 17-AAG on EGFR expression was observed between 3 and 12 h with recovery of EGFR to baseline levels of expression by 48 h. The effect of 17-AAG on the activity of the EGFR pathway, as assessed by the expression of phosphorylated MAPK and Akt and cyclin D1 expression, however, was less durable with recovery observed by 12 h (Fig. 3 and data not shown). The Hsp90 client proteins Raf-1 and Akt were notably less sensitive to 17-AAG–induced degradation in these models. Specifically, Raf-1 was only partially degraded by 17-AAG (55% down-regulation at 6 h), and Akt expression was unaffected by 17-AAG treatment in these model systems (Fig. 3). Finally, treatment with 17-AAG had no effect on the expression of wild-type EGFR in A549 xenografts. This insensitivity of wild-type EGFR to 17-AAG was not due to the inability of the compound to inhibit Hsp90, as HER2 was potently degraded by 17-AAG in the A549 xenografts. These results highlight the wide variability in sensitivity *in vivo* of the various Hsp90 clients.

As 17-AAG could effectively down-regulate the expression of mutant forms of EGFR in xenograft tumors at nontoxic doses, we assessed the antitumor effects of chronic treatment with 17-AAG in

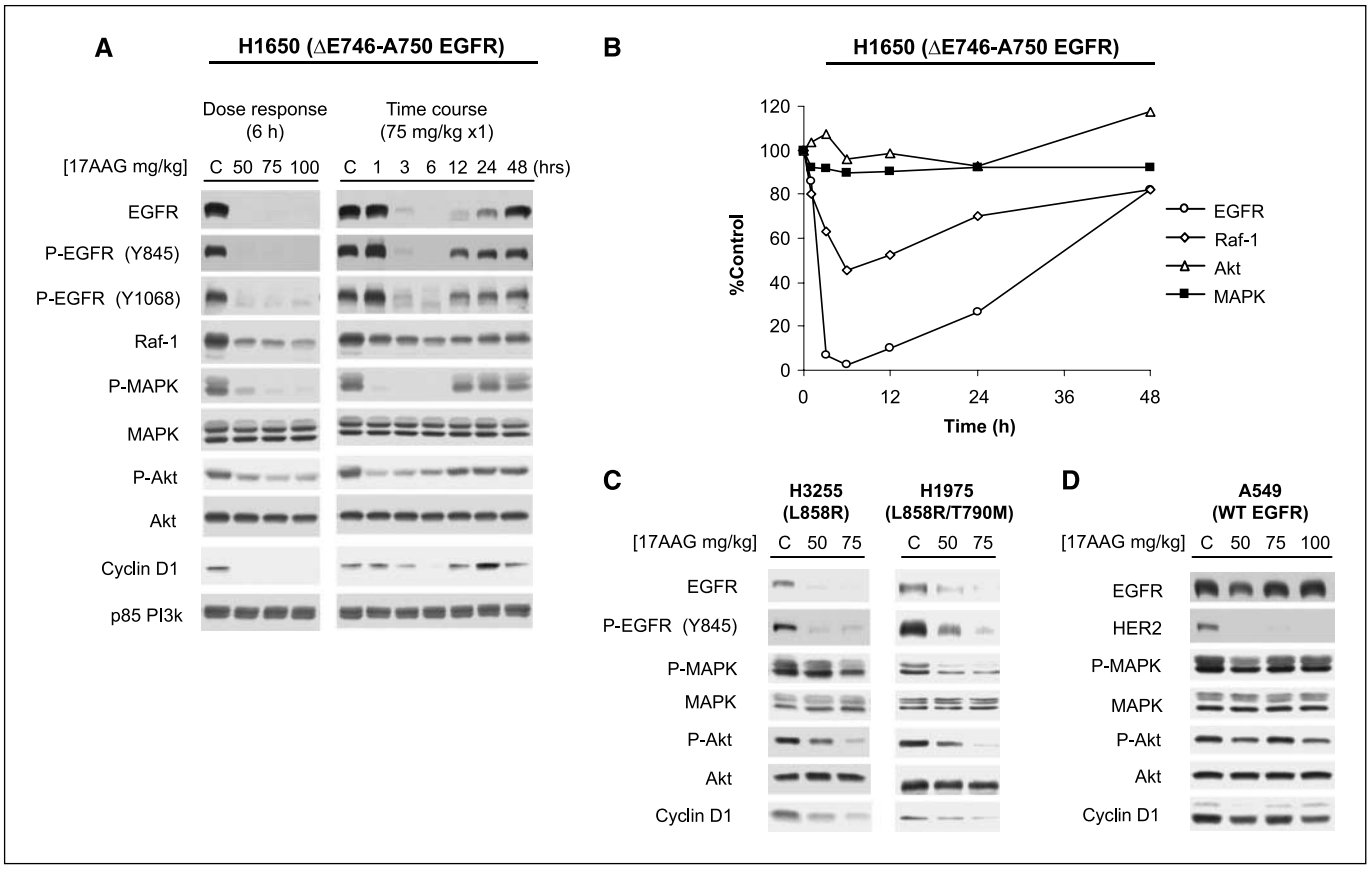

Figure 3. 17-AAG, at nontoxic doses, down-regulates mutant EGFR expression in xenograft tumors. For dose-response studies, mice were treated with either 50 or 75 mg/kg of 17-AAG and sacrificed 6 h later. In time course experiments mice were treated with a single dose of 75 mg/kg of 17-AAG and sacrificed at the time points indicated (0–48 h). *A,* immunoblots of EGFR, Raf-1, Akt, and cyclin D1 in H1650 xenografts treated with 17-AAG. 17-AAG treatment resulted in a >95% reduction in mutant EGFR expression and inhibition of AKT and MAPK activity in H1650 xenografts. Raf-1 was less sensitive than mutant EGFR to 17-AAG treatment, and Akt expression was unaffected by 17-AAG. *B,* quantitation of the effects of 17-AAG on the expression of EGFR, Raf-1, and Akt in H1650 xenografts. MAPK, a protein unaffected by Hsp90 inhibition, is included as a loading control. *C,* similar results were observed in H3255 and H1975 xenografts (*C* and data not shown). *D,* 17-AAG does not induce the degradation of wild-type EGFR but does down-regulate the expression of HER2 in A549 xenografts.

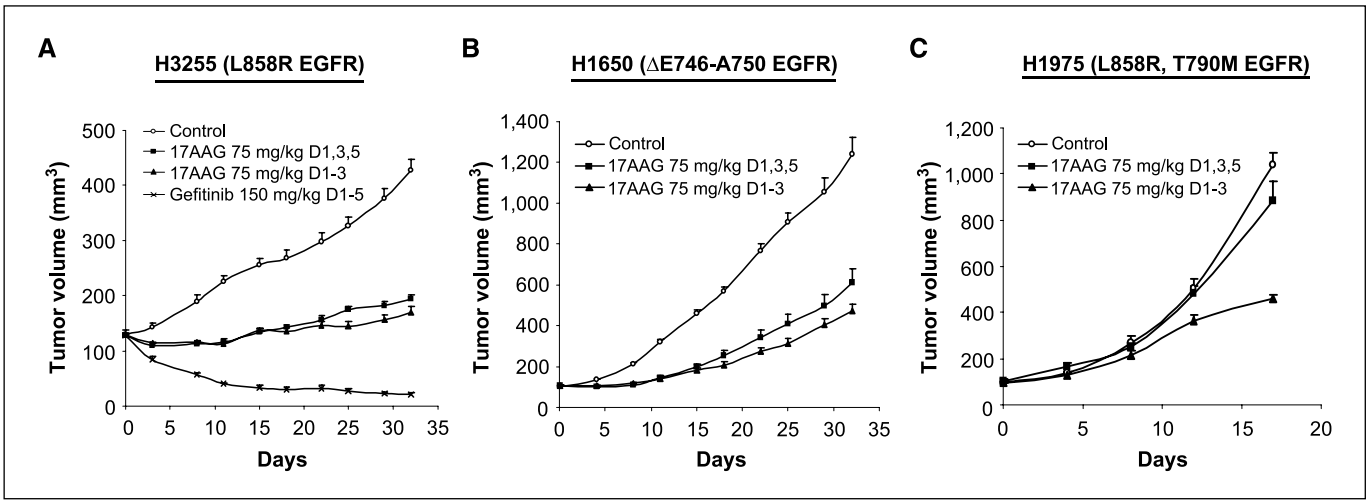

Figure 4. 17-AAG inhibits the growth of EGFR mutant lung cancer xenogafts. Mice were treated with 17-AAG using one of two schedules: 75 mg/kg three times per week either on alternating days (days 1, 3, and 5) or consecutive days (days 1–3; $n = 5$ mice per group). Only mice with H3255 xenografts were treated with gefitinib at 150 mg/kg, 5×/wk (days 1–5). *A,* 17-AAG significantly delayed the growth of H3255 but was less effective in this model than gefitinib, which induced significant tumor regression ($P < 0.01$ for the comparison of 17-AAG to control or 17-AAG to gefitinib). *B and C,* 17-AAG alone inhibited the growth of H1650 and H1975 xenografts. Mice were randomized, and treatment was initiated at day 0. *Bars,* SE.

mice with established H3255 (L858R EGFR), H1650 (ΔE746_A750 EGFR), H1975 (L858R, T790M EGFR), and A549 (wild-type EGFR) xenografts (Fig. 4 and Supplementary Fig. S3). H3255 is sensitive to the receptor TKI gefitinib (IC_{50} of 0.1 nmol/L), whereas H1650 and H1975 are resistant to gefitinib (IC_{50}s > 10 μmol/L). Mice were treated with 75 mg/kg of 17-AAG using one of two schedules: alternating days (days 1, 3, and 5 each week) or three consecutive days (days 1–3) each week. These schedules were chosen based upon our prior studies showing that more frequent dosing schedules of 17-AAG were unacceptably toxic (11, 33). Mice with established H3255 (L858R EGFR, gefitinib sensitive) xenografts were also treated with gefitinib at a dose of 150 mg/kg (days 1–5 each week). 17-AAG treatment of H3255-bearing mice resulted in marked growth delay ($P = 0.01$) but was less effective than gefitinib, which induced significant tumor regression ($P < 0.01$). 17-AAG was less active in H1650-bearing and H1975-bearing mice and had no activity in the EGFR wild-type A549 model (Fig. 4 and Supplementary Fig. S3). As the H1650 and H1975 models are resistant to gefitinib, the relative resistance of these models to 17-AAG may be attributable to additional genetic alterations, which reduce their dependence upon EGFR. For example, H1650 cells do not express the tumor suppressor PTEN, a genetic alteration associated with resistance to EGFR TKIs in patients with glioblastoma (34, 35).

17-AAG sensitizes EGFR mutant lung adenocarcinoma xenografts to paclitaxel. As outlined above, 17-AAG can effectively down-regulate mutant EGFR expression and inhibit the growth of EGFR mutant xenografts. However, because of toxicity limitations and its short half-life, continuous EGFR inhibition *in vivo* was not possible with this agent. As 17-AAG has previously been shown by us and others to enhance the activity of cytotoxic agents (33, 36, 37), we asked whether 17-AAG could enhance the activity of paclitaxel in lung cancer xenografts with EGFR mutations. Mice with H1650 and H1975 xenografts were treated with 25 mg/kg of paclitaxel weekly (on day 1 each week) and/or 75 mg/kg of 17-AAG (on days 1–3 each week). Control mice were treated with the vehicles alone. In mice bearing H1650 and H1975 xenografts, the combination of paclitaxel and 17-AAG was more effective than either agent alone (Fig. 5). To further assess the mechanism of this effect, mice were sacrificed pretreatment and 24 and 48 h after the start of paclitaxel, 17-AAG, or the combination, and the tumors were flash frozen. 17-AAG down-regulated the expression of mutant EGFR in both the 17-AAG alone and 17-AAG/paclitaxel combination arms (Fig. 6). Paclitaxel alone had no effect on mutant EGFR expression. Apoptosis as measured by PARP cleavage was observed in both the 17-AAG alone and 17-AAG/paclitaxel arms but not in mice treated with paclitaxel alone. Notably, the duration of this effect was longer in mice treated with the combination (Fig. 6). These data suggest that 17-AAG can be safely combined with paclitaxel and that the combination may be a more effective treatment strategy than the use of 17-AAG alone in patients whose tumor express mutant forms of EGFR.

Discussion

Hsp90 is an abundant cellular chaperone that is required for refolding of unfolded proteins, cellular survival under stress conditions, and the conformational maturation of a variety of proteins that play key roles in transducing proliferative and antiapoptotic signals. The clinical development of inhibitors of Hsp90 has been based upon two fundamental preclinical observations. First, we and others have shown that inhibition of Hsp90 is selectively toxic to tumor cells (38, 39). In tumor cells, most Hsp90 is found in an active complex with cochaperones, whereas most Hsp90 in normal tissues exists in a free, uncomplexed, or latent state (39). Hsp90 in the active, cochaperone bound complex has a higher affinity for geldanamycin and other Hsp90 inhibitors compared with free, uncomplexed Hsp90 (38, 39). These data likely explain in part the accumulation of 17-AAG and other Hsp90 inhibitors in tumors. These observations also suggest that Hsp90 may be "limiting" in tumor cells and may thus explain in part the greater sensitivity of tumor versus normal cells to Hsp90 inhibitors. One prediction based upon these data is that Hsp90 inhibitors may have broad antitumor activity in a diverse range of cancer types. The finding that Hsp90 is limiting in tumor cells may

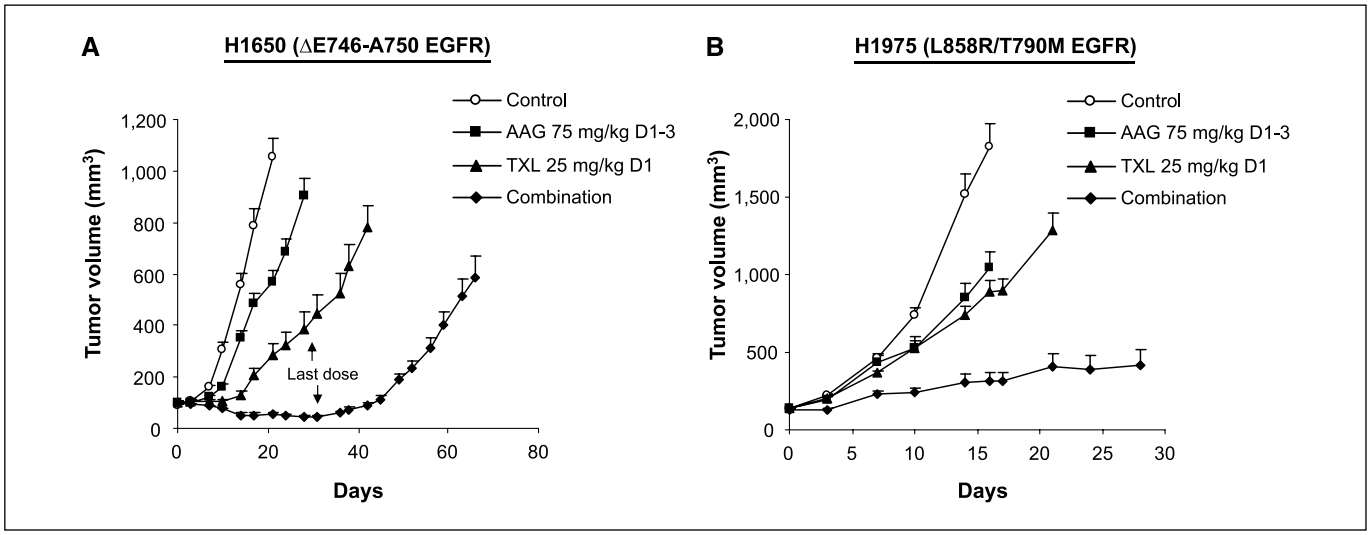

Figure 5. Inhibition of Hsp90 by 17-AAG sensitizes H1650 and H1975 xenografts to paclitaxel. Mice with established H1650 (*A*) and H1975 (*B*) xenografts were treated with paclitaxel weekly (day 1) at a dose of 25 mg/kg and with 17-AAG for three consecutive days each week (days 1–3). Control mice were treated with the vehicles alone. Mice were treated with the drugs for a maximum of 30 d and then monitored. In mice with established H1650 xenografts, the combination of paclitaxel and 17-AAG was significantly more effective than either 17-AAG or paclitaxel alone ($P = 0.02$ for paclitaxel/17-AAG versus paclitaxel). Five mice were treated per group with the results then confirmed with a second cohort of mice (data not shown). Mice were randomized, and treatment was initiated at day 0. *B,* the combination of 17-AAG and paclitaxel was also more effective than either drug alone in mice with established H1975 tumors. *Bars,* SE.

also explain the synergy observed between inhibitors of Hsp90 and chemotherapy, radiation, and inducers of hypoxic stress.

A competing model is that Hsp90 inhibitors will have activity only in a small subset of tumor types in which key aspects of transformation are dependent upon a sensitive Hsp90-dependent client, such as mutant EGFR. In support of this latter possibility, we find significant variability in the sensitivity of individual Hsp90 client proteins to degradation by Hsp90 inhibitors. Whereas over 100 transcription factors, kinases, steroid receptors, and other regulatory proteins have been shown to interact with Hsp90, our animal studies suggest that only a fraction of these Hsp90 clients are in fact degraded by Hsp90 inhibitors *in vivo* at nontoxic doses. The mechanistic basis for this hierarchy of client sensitivity (i.e., the sensitivity of HER2 > mutant EGFR > Raf-1 > Akt > mutant BRAF> wild-type EGFR) remains to be defined but may be due to intrinsic difference between clients (i.e., client half-life) or to differences in their binding to individual Hsp90 family members or cochaperones. Identification of the subset of "most sensitive" Hsp90 clients may thus aid in choosing those patients most likely to benefit from an Hsp90 inhibitor.

With this goal in mind, we compared the sensitivity of wild-type and mutant EGFR to down-regulation by the Hsp90 inhibitor 17-AAG. We chose 17-AAG for these studies, as it is currently being tested in human clinical trials. We observed that both an exon 19 deletion mutant and the L858R exon 21 mutant were more sensitive to 17-AAG–induced degradation than wild-type EGFR. In studies using isogenic cells stably expressing either the wild-type or mutant forms of EGFR, the mutant forms were degraded faster and by lower concentrations of the inhibitor than the wild-type protein. We further show, using xenograft model systems, that 17-AAG could induce the down-regulation of mutant forms of EGFR but not the wild-type protein in xenograft tumors at nontoxic doses and that this was accompanied by antitumor effects in the EGFR mutant models. The expression of Akt, which is also an Hsp90 client protein, was unaffected by treatment with nontoxic doses of 17-AAG. However, the activity of Akt, as measured by the expression of Ser^{473} phosphorylated AKT, was down-regulated by 17-AAG treatment. Inhibition of Akt activity by 17-AAG may be of particular importance in this context, as Akt activation has been shown to be EGFR-dependent and to promote cell survival in lung cancer cell lines with EGFR mutations (40).

Notably, treatment with 17-AAG at its maximally tolerated dose could only slow tumor growth in the H3255 (L858R EGFR) xenograft model, and 17-AAG was less effective in this model than the EGFR TKI gefitinib, which was capable of inducing complete tumor regressions. The likely explanation for the limited efficacy of 17-AAG in this setting is that continuous down-regulation of

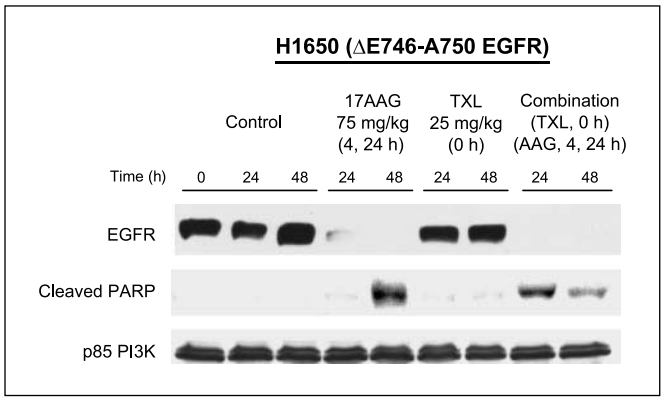

Figure 6. Induction of apoptosis by the combination of paclitaxel and 17-AAG in H1650 xenografts. Immunoblots of EGFR, cleaved PARP, and p85 PI3K showing that 17-AAG alone and the combination of paclitaxel and 17-AAG induced apoptosis as shown by PARP cleavage. However, the duration of apoptosis was longer in the combination-treated mice compared with those treated with 17-AAG alone. Apoptosis was not observed after treatment with paclitaxel alone. p85 PI3K, a protein unaffected by Hsp90 inhibition, was included as a loading control. Results were repeated and confirmed with a second set of mice (data not shown). Mice were treated with paclitaxel (25 mg/kg) at time zero or with 17-AAG at 4 and 24 h at a dose of 75 mg/kg. Mice in combination group were treated with paclitaxel at 0 h and with 17-AAG at 4 and 24 h.

mutant EGFR and EGFR signaling could not be achieved in mice. The inability to continuously down-regulate EGFR activity is likely attributable to the short half-life of 17-AAG in mice and the inability to dose this agent chronically on a daily schedule due to its hepatotoxicity (11). Our data, thus, provide a strong rationale for the development of novel Hsp90 inhibitors with improved oral bioavailability and less hepatotoxicity, which can be dosed using a daily continuous schedule. Several synthetic small molecule Hsp90 inhibitors with these characteristics have now been developed, and phase 1 trials of two such agents, CNF-2024 and SNX-5422, have begun (38, 41).

Although the activity of 17-AAG as a single agent in EGFR mutant tumors was modest, we did observe that 17-AAG and paclitaxel could be safely combined without additive toxicity and that the combination was more effective than either agent alone in mice with established H1650 and H1975 tumors. We and others have previously shown using multiple model systems, including HER2-dependent breast and ovarian cancers, that the combination of 17-AAG and paclitaxel is synergistic (37, 42, 43). In breast cancers with HER2 amplification, enhancement is schedule dependent and mediated by 17-AAG–induced down-regulation of Akt activity (33). Notably, continuous inhibition of Hsp90 by 17-AAG was not required in these studies to achieve a maximal effect with the combination. In studies of the EGFR TKI gefitinib, pulsatile dosing was also shown to be superior to continuous treatment when combining this agent with paclitaxel (44). Therefore, whereas the efficacy of 17-AAG as a single agent may be limited by its inability, because of toxicity, to continuously suppress Hsp90 function, the full potential benefits of Hsp90 inhibition may be clinically achievable when this inhibitor is used on an intermittent basis to sensitize tumors to cytotoxics, such as paclitaxel.

In summary, our data suggest that Hsp90 inhibitors may be an effective clinical strategy in patients whose tumors are dependent upon mutant forms of EGFR. Our data, however, suggest that 17-AAG alone may have only limited activity in such patients and that combination therapy with a cytotoxic may be a more effective clinical strategy. Based upon these data, we initiated a phase 1 trial of docetaxel and 17-AAG in patients with advanced cancer. In this study, the two agents could be safely coadministered every 3 weeks, and promising clinical activity has been observed on this trial in patients with NSCLC (45). We therefore propose further studies of Hsp90 inhibitors in combination with taxanes in lung cancer patients whose tumors express activating mutations of EGFR.

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