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. 

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 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
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 Confora 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 room temperature. Gefitinib was obtained from AstraZeneca Cancer Institute. Paclitaxel was obtained from Bristol-Myers Squibb and stored in a 1:1 mixture of DME:F-12, A549 in F12K, and NCI-H441, H1650, H1666, AmericanTypeCultureCollection. NCI-H3255 cells were provided by Drs. B.

lysedinNP40lysisbuffer[50mmol/LTris(pH7.4),1%NP40,150mmol/L

and Supplementary Fig. S1). In addition to the L858R mutant forms of EGFR in lung adenocarcinoma cell lines (Fig. 2

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).

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. L4 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
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...
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. 3C 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...
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 (IC₅₀ of 0.1 nmol/L), whereas H1650 and H1975 are resistant to gefitinib (IC₅₀ > 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). 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 loss of binding 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
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 Ser473 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
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 sensitizes 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.

Acknowledgments


Grant support: National Cancer Institute grants P50-CA92629 and P01 CA94060 and the Byrne Fund.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. We thank William Pao, Yixin Gong, Bruce Johnson, and Pas Janni for providing cell lines and reagents, Elisa DeStanchina, Wai Lin Wong, and Juan Qiu for assistance with the xenograft studies, Francis Burrows, Marcus Boehm, and Larry Fritz of Conformia Therapeutics for providing 17-AAG, and Cancer Therapy Evaluation Program of National Cancer Institute for providing the EPL diluent.

References


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