Epidermal Growth Factor Receptors Harboring Kinase Domain Mutations Associate with the Heat Shock Protein 90 Chaperone and Are Destabilized following Exposure to Geldanamycins

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Abstract

Somatic mutations in the kinase domain of the epidermal growth factor receptor (EGFR), including L858R and exon 19 deletions, underlie responsiveness to gefitinib and erlotinib in non–small cell lung cancer (NSCLC). Acquired resistance to these tyrosine kinase inhibitors is in some cases mediated by a second mutation, T790M. Ansamycin antibiotics, such as geldanamycin, potently inhibit heat shock protein 90 (Hsp90), promoting ubiquitin-mediated degradation of oncogenic kinases that require the chaperone for proper conformational folding. Here, we show that L858R and deletion mutant EGFR proteins found in NSCLC interact with the chaperone and are sensitive to degradation following Hsp90 inhibition. In NIH/3T3 cells expressing either wild-type or mutant EGFR, diminution of expression of both L858R and EGFR delE747-S752, P753S occurred following exposure to 50 nmol/L geldanamycin over 24 hours, whereas partial diminution of wild-type EGFR following exposure to 50 nmol/L geldanamycin over 24 hours, whereas partial diminution of wild-type EGFR was less substantial and seen only following 12 hours. Similarly, EGFR proteins in NSCLC cell lines harboring EGFR mutations, including NCI-H1650, NCI-H3255, and NCI-H1975, were also more sensitive to geldanamycin-induced degradation compared with the protein in wild-type cells. Exposure of EGFR-mutant cell lines to geldanamycin induced marked depletion of phospho-Akt and cyclin D1 as well as apoptosis. These data suggest mutational activation of EGFR is associated with dependence on Hsp90 for stability and that Hsp90 inhibition may represent a novel strategy for the treatment of EGFR-mutant NSCLC. (Cancer Res 2005; 65(14): 6401-8)

Introduction

Activating mutations in the kinase domain of the epidermal growth factor receptor (EGFR) occur in ~10% of non–small cell lung cancers (NSCLC) from the United States and in a higher percentage of tumors isolated from Asian populations (1–3). These mutations are usually small exon 19 deletions, or point mutations, most commonly a replacement of leucine by arginine at codon 858 (L858R) in exon 21. Mutant EGFRs induce oncogenic effects by activating downstream signaling and antiapoptotic pathways, most notably those mediated by signal transducer and activator of transcription (STAT) proteins and Akt (4). Both types of mutation confer sensitivity to the anilinoquinazoline inhibitors of EGFR, gefitinib and erlotinib, likely by repositioning critical residues of the tyrosine kinase domain, stabilizing their interactions with ATP as well as with these ATP-competitive inhibitors (1–3).

Despite the initial dramatic responses of EGFR-mutant tumors to small-molecule tyrosine kinase inhibitors, resistance universally emerges over time. Acquired resistance has recently been shown to be associated with a second somatic mutation, resulting in a threonine-to-methionine amino acid substitution at position 790 (T790M) of EGFR (5, 6), analogous to the T315I mutation that confers resistance of BCR-ABL to imatinib (7, 8). Therefore, the investigation of second-generation targeted agents for this subset of lung cancers is warranted. This could involve the development of drugs capable of inhibiting EGFR despite the T790M mutation or of drugs targeting downstream phosphatidylinositol 3’-kinase (PI3K) or STAT5 pathways.

An alternative strategy, potentially relevant to NSCLCs harboring EGFR mutations both sensitive and resistant to anilinoquinazoline inhibitors, is to target mutant EGFR for degradation. Many kinases that contribute to deregulated signaling and proliferation in human cancers rely on the heat shock protein 90 (Hsp90) chaperone for conformational maturation (9). Hsp90 is a member of the heat shock protein/chaperone family, a family that assists in the folding of newly synthesized proteins in the cell as well as in protein refolding after environmental insults (10). Some of the kinases dependent on Hsp90 for maturation and stability include the HER2 (ErbB2) and c-met receptor tyrosine kinases, the v-Src family of non–receptor tyrosine kinases, and the serine/threonine kinases Raf1, Akt, and cyclin-dependent kinase 4 (cdk4; ref. 11). Benzoxquinoid ansamycins, including herbimycin A and geldanamycin, bind to a conserved pocket in the Hsp90 chaperone (12) and prevent its association with client proteins, causing them to undergo ubiquitin-mediated proteasomal degradation (13). For example, in many NSCLCs as in other tumor types, we have shown that depletion of cdk4 by geldanamycin contributes to retinoblastoma (Rb)–dependent G1 arrest and cytostatic effects on tumor cell growth (14–16). In BRAF, mutation confers greater dependence on the chaperone for proper folding, such that mutant proteins are more sensitive than wild-type to degradation following exposure to Hsp90 inhibitors (17). In addition, BCR-ABL is degraded after Hsp90 inhibition; importantly, imatinib-resistant BCR-ABL point mutants remain sensitive to Hsp90 inhibitors (18).
HER2 and wild-type EGFR respond very differently to geldanamycin. Hsp90 can be found in HER2 immunoprecipitates, indicating that HER2 is associated with the chaperone (19). Following exposure to geldanamycin, HER2 is depleted within 4 hours from SKBR3 HER2-overexpressing breast cancer cells. However, coprecipitation experiments have not revealed an association of EGFR with Hsp90. Exposure of EGFR-overexpressing A-431 cells to geldanamycin results in a much slower depletion of EGFR, requiring 16 to 20 hours, with a time course identical to that seen following treatment with cyclohexamide (19). These results suggest that the effects of geldanamycin on EGFR are mediated solely by its ability to destabilize newly synthesized protein; once nascent receptors are translocated into the endoplasmic reticulum membrane, addition of Hsp90 inhibitors does not compromise their stability (20). In contrast, the association of mature HER2 receptors with Hsp90, as well as exquisite sensitivity to geldanamycin, is maintained (19, 21). In addition, in contrast to wild-type EGFR, EGFRIlll has been found to associate with Hsp90 (22). This variant, found in glioblastomas (23) and also described in breast and lung carcinomas (24), carries a deletion of exons 2 to 7 causing single amino acids 6 to 273 of the extracellular domain to be replaced by a single glycine residue and is relatively resistant to inhibition by gefitinib (25). Expression of EGFRIlll is compromised following exposure of glioblastoma cells to geldanamycin and the chemically unrelated radicicol, which also targets Hsp90 (22).

These observations prompted us to examine the sensitivity to geldanamycin of EGFR harboring mutations in the kinase domain recently identified in NSCLC. Here, we show that receptors carrying the E746-A750 deletion or the L858R point mutation with or without the secondary T790M mutation associate with Hsp90 and are rapidly depleted from EGFR-mutant NSCLC cell lines, resulting in apoptosis. Because the mutational activation of EGFR is associated with dependence on Hsp90 for maintenance of stability, the use of geldanamycin derivatives may represent a promising strategy for the treatment of EGFR-mutant NSCLC.

**Materials and Methods**

**Tumor cell lines.** NCI-H1650, NCI-H1975, NCI-H1666, Calu-1, A549, NCI-H520, NCI-H441, and NCI-H538 NSCLC cell lines and A-431 epidermoid carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD). All cell lines were maintained in the cell growth medium specified by American Type Culture Collection. NCI-H225 cells (2, 26) were supplied by Drs. Bruce Johnson and Pasi Ja¨nne (Dana-Farber Cancer Institute, Boston, MA) and grown in ACL-4 medium supplemented with 10% fetal bovine serum and penicillin-streptomycin.

**Epidermal growth factor receptor constructs and NIH/3T3 cells.** Human EGFR was cloned into pDNR-Dual (BD Biosciences, Mountain View, CA). The deletion mutant, L747-S752del, P753S was constructed using the following oligonucleotides: sense CCGTCGCTATTGGGTGCTGGCCG and antisense CCCAGCAGTTTGGCCCGCCT. Directed Mutagenesis (Stratagene, La Jolla, CA). The L858R mutation was constructed according to Quick Change Site-Directed Mutagenesis (Stratagene, La Jolla, CA) and ethanol fixation, cells were incubated with fluorescein-12 dUTP in the presence of TdT. Error bars represent the SE generated over a minimum of three independent experiments.

**Drug treatment.** Stock solutions of geldanamycin and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17DMAG) were prepared in DMSO at a concentration of 10 mmol/L and maintained at −20°C. Drugs were diluted to 1 mmol/L in DMSO for a working solution and used at concentrations ranging from 50 to 1,000 mmol/L. Geldanamycin was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, MD). 17DMAG was obtained from Invivogen (San Diego, CA).

**Western blot analysis.** Whole-cell lysates were prepared in NP40 lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP40] supplemented with protease and phosphatase inhibitor I and II cocktails (Calbiochem, San Diego, CA) and clarified by centrifugation. Protein concentrations were determined using the Bicinchoninic Acid Protein Assay kit (Pierce, Rockford, IL) and equivalent amounts (40 μg) were subjected to SDS-PAGE on 12% gels, except where indicated. Western blotting was done as described previously (29). Anti-EGFR, phospho-EGFR (Tyr1092/1093), Akt, extracellular signal-regulated kinase 1/2 (Erk1/2), anti-cyclin D1 antibody (DCS-6) was from EMD Biosciences (San Diego, CA). The anti-Hsp90 antibody was from Stressgen (Victoria, British Columbia, Canada). Anti-α-tubulin (clone DM 1A) and the anti-RasGAP antibodies, used as an equal loading controls, were purchased from Sigma-Aldrich Co. (St. Louis, MO) and from Upstate Biotechnology (Lake Placid, NY), respectively.

**Immunoprecipitation.** For the detection of EGFR and Hsp90 complex, whole-cell lysate (1 μg) in NP40 lysis buffer was incubated with Agarose A/G Plus preconjugated with anti-EGFR rabbit IgG (SC-3, Santa Cruz Biotechnology). Immunoprecipitates were washed in NP40 lysis buffer, boiled in sample buffer, and subjected to SDS-PAGE followed by Western blotting using an anti-Hsp90 antibody to detect complex formation. Recovery of EGFR was monitored by Western blotting.

**Cell proliferation assay.** Cells (5 × 10⁴ per well) were seeded on 96-well plates and allowed to grow in appropriate growth medium in the presence or absence of drug for 72 hours. Cell metabolic activity was determined every 24 hours by using the CCK-8 (Dojindo, Gaithersburg, MD) colorimetric assay according to the manufacturer’s instructions. Results were expressed as percent of viable cell number as determined by trypan blue exclusion. Error bars represent the SE generated over three experiments in which each condition was assayed in triplicate.

**Fluorescence-activated cell sorting analysis.** Cell cycle analysis was done as described previously (29). Nonadherent and adherent cells were combined following fixation and treatment in 500 μg/ml RNase A, cells were resuspended in 69 mmol/L propidium iodide (1 mL) in 30 mmol/L sodium citrate. Cells were analyzed for DNA content by flow cytometry using the ModFit program (Verity Software House, Topsham, ME).

**Detection of apoptosis by flow cytometry.** A fluorescein apoptosis detection kit was used (Promega, Madison, WI) as described previously (29). Nonadherent and adherent cells were combined. Following formaldehyde and ethanol fixation, cells were incubated with fluorescein-12 dUTP in the presence of terminal deoxynucleotidyl transferase (TdT). Following washes described in the manufacturer’s instructions, cells were resuspended in PBS containing 5 μg/ml propidium iodide and 500 μg/ml RNase A. Cells were analyzed for DNA content and apoptosis using two-color flow cytometry. Apoptosis was quantitated as the percent of cells shifting to fluorescein positive in the presence of TdT. Error bars represent the SE generated over a minimum of three independent experiments.

**Results**

Mutant epidermal growth factor receptors are more sensitive than wild-type to heat shock protein 90 inhibition mediated by geldanamycins. To determine whether mutant EGFR proteins are more sensitive than wild-type to Hsp90 inhibition, we used NIH/3T3 cells engineered to constitutively express wild-type EGFR, EGFR with the L858R point mutation, or EGFR harboring the L747-S752, P753S exon 19 deletion. As shown...
in Fig. 1A, similar levels of exogenous EGFR are expressed in these cell lines. In addition, levels of Hsp90 are comparable in cells expressing empty vector (GFP) and in those expressing either wild-type or mutant EGFR proteins. Known client proteins of Hsp90, including c-Raf and Akt, are similarly depleted in these cell lines in a concentration-dependent manner following 24-hour exposure to geldanamycin. However, wild-type and mutant EGFRs behave differently in response to geldanamycin. Although the expression of wild-type EGFR is compromised, its levels remain high even after exposure to 200 nmol/L drug. In contrast, substantial depletion of mutant EGFR expression is evident following treatment with 50 nmol/L geldanamycin and is undetectable after 24-hour exposure to 200 nmol/L drug. These results were confirmed with 17DMAG, a geldanamycin derivative suitable for clinical use (Fig. 1B).

To further investigate the differences between mutant and wild-type EGFRs, time course experiments were done. In the experiment shown in Fig. 2, cells were exposed to 1 μmol/L geldanamycin for up to 12 hours, and levels of expression were determined at 2-hour intervals. Again, across these cell lines, known clients of Hsp90, including Akt and cdk4, were equally diminished in a time-dependent manner. Partial diminution of wild-type protein was evident by 10 hours, consistent with the slow depletion of wild-type protein described previously in A-431 cells exposed to geldanamycin. In contrast, expression of mutant EGFR proteins was decreased >50% by 4 hours and was nearly undetectable by 6 hours, consistent with greater sensitivity to the disruption of chaperone function.

**Mutant epidermal growth factor receptors in non–small cell lung cancer cell lines are more sensitive to degradation than wild-type epidermal growth factor receptor following geldanamycin-mediated heat shock protein 90 inhibition.**

We next extended our results to NSCLC cell lines. In this analysis, we used NCI-H1650 (del E746-A750), NCI-H3255 (L858R), and NCI-H1975 (L858R + T790M), whereas other cell lines analyzed expressed wild-type EGFR. Cell lines expressed similar levels of Hsp90. At the concentrations of geldanamycin used, known Hsp90 client proteins, including c-Raf and Akt, showed a similar reduction (Fig. 3), with the exception of cdk4 in A549 cells (data not shown). These results suggest that drug uptake was similar among these cell lines. As was the case in engineered NIH/3T3 cells, mutant EGFR was depleted from NCI-H1650, NCI-H3255, and NCI-H1975 cells following exposure to 50 nmol/L geldanamycin, whereas wild-type EGFR expression persisted at concentrations as high as 500 nmol/L drug (Fig. 3A and B). As shown in Fig. 3C, phospho-EGFR was depleted in parallel with total EGFR in the cell lines examined. These data confirm that activating kinase domain mutation is associated with chaperone dependence; in NCI-H1975 cells, chaperone dependence is maintained in the presence of a second mutation conferring EGFR inhibitor resistance.

**Mutant epidermal growth factor receptor associates with the heat shock protein 90 chaperone.** To further corroborate the dependence of EGFR L858R and del E746-A750 on the chaperone, we sought evidence of the association of these mutant proteins with Hsp90. As shown in Fig. 3D, Hsp90 could be detected in immunoprecipitates of EGFR from NCI-H3255, NCI-H1650, and NCI-H1975 cells and only very weakly in A-431 cells. NCI-H520 cells, which express very low levels of wild-type EGFR, were used as an additional negative control. Again, the results with NCI-H1975 cells indicate that association with the chaperone occurs in the presence of the T790M mutation.

**Inhibition of downstream phosphorylation of Akt and extracellular signal-regulated kinase 1/2 occurs following geldanamycin-induced depletion of mutant epidermal growth factor receptor in non–small cell lung cancer cell lines.** Inhibition of mutant EGFR using anilinoquinazoline inhibitors results in rapid depletion in phospho-Akt and phospho-Erk1/2 (2, 4). To confirm that similar events occur after exposure to geldanamycin, we examined levels of these proteins following

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**Figure 1.** Mutant EGFRs are more readily depleted than wild-type EGFR following Hsp90 inhibition by geldanamycins. A, control cells (JP1520) and NIH/3T3 cells constitutively expressing wild-type or mutant EGFR were treated with the indicated concentrations of geldanamycin for 24 hours. Lysates were subjected to Western blotting with the indicated antibodies, showing that mutant EGFRs are more sensitive to geldanamycin treatment than wild-type EGFR, whereas c-Raf and Akt respond similarly in these cell lines. B, similar experiment with 17DMAG, confirming the results with geldanamycin.
treatment (Fig. 4A). As Akt is an Hsp90 client protein, total Akt depletion was observed in both EGFR-mutant and wild-type cells (30, 31). Although phospho-Akt levels paralleled total Akt levels in wild-type cells, phospho-Akt levels were reduced more rapidly than total Akt in EGFR-mutant cell lines (Fig. 4A and B). Therefore, the rapid depletion of phospho-Akt from these cells is most likely a result of depletion of mutant EGFR, which occurs following exposure to similar geldanamycin concentrations rather than a reflection of the less pronounced reduction in total Akt level induced by Hsp90 inhibition. Similarly, phospho-Erk was also rapidly depleted from mutant cell lines, again consistent with the destabilization of EGFR proteins in these cells.

Depletion of cyclin D1 and cyclin-dependent kinase 4 following exposure to geldanamycin in non–small cell lung cancer cell lines. cdk4 is an Hsp90 client (16) that is depleted following exposure to geldanamycins in NSCLC cell lines as well as in other cell types and is associated with Rb-dependent G1 arrest. However, in our previous survey of NSCLC cell lines treated with geldanamycin derivatives, cyclin D1 levels were stable following 24-hour exposure (14). However, in some cellular contexts, cyclin D expression is controlled by a PI3K-Akt dependent pathway (32). We therefore examined whether expression of cyclin D1 was compromised following geldanamycin exposure in EGFR-mutant lines (Fig. 4C). In contrast to effects observed in EGFR wild-type cells, rapid depletion of cyclin D1 occurred in NCI-H3255 and NCI-1975 cells and to a lesser extent in NCI-H1650 cells (Fig. 4C and D).

Geldanamycin induces apoptosis in epidermal growth factor receptor–mutant non–small cell lung cancer cell lines. Cell proliferation assays were done in the variety of EGFR-mutant and wild-type NSCLC cell lines (Fig. 5A). These assays showed a
decrease in viable cell number compared with control-treated cells over a 48- to 72-hour exposure. In the majority of wild-type cell lines, this is due to decreased proliferation secondary to cell cycle arrest at the G1-S and G2-M boundaries (Fig. 5B). In contrast, as shown in Fig. 5B and C, apoptosis was evident in EGFR-mutant lines, with the appearance of sub-G1 peak; DNA fragmentation was confirmed by a flow cytometry–based TdT-mediated dUTP nick end labeling (TUNEL) assay, including cell lines exquisitely sensitive (NCI-H3255) or more resistant (NCI-H1975 and NCI-H1650) to anilinoquinazoline inhibitors. Results were confirmed by Western blotting, showing evidence of poly(ADP-ribose) polymerase (PARP) cleavage following geldanamycin treatment in these cells (Fig. 5D).

Discussion

EGFR-mutant NSCLCs have shown dramatic responses to the anilinoquinazoline inhibitors, in part related to the altered sensitivity of mutant proteins to these inhibitors. In addition, these responses are likely also related to “oncogene addiction” in which cell death occurs following suppression of an oncogenic signal on which they have become dependent (33). Consistent with the dependence of this subset of lung adenocarcinomas on EGFR, EGFR mutation stimulates PI3K-Akt and STAT pathways that promote cell survival, and small interfering RNA (siRNA)–mediated depletion of EGFR expression in mutant cell lines results in rapid loss of viability and apoptosis (4).

Acquired resistance to the anilinoquinazoline EGFR inhibitors is universal and in some cases mediated by the development of or selection for a second mutation in EGFR that confers resistance to these drugs. The appearance of such second mutations suggests that the tumor cells remain dependent on an active EGFR pathway for their survival. Indeed, siRNA-mediated depletion of EGFR from NCI-H1975 cells, which contain both the L858R point mutation and the T790M mutation, induces apoptosis, as occurs in mutant cell lines not reported to carry T790M (4).

The emergence of tumor cells resistant to the anilinoquinazoline inhibitors that retain EGFR dependence indicates that alternative strategies targeting EGFR or downstream pathways may have antitumor activity in this lung cancer subtype. Here, we have shown that EGFRs harboring kinase domain mutation interact with the Hsp90 chaperone and are rapidly depleted from cells following Hsp90 inhibition, suggesting that they are dependent on chaperone function for conformational maturation and stability. The depletion of mutants was shown for receptors carrying an exon 19 deletion (NCI-H1650) as well as those carrying the L858R point mutation either with (NCI-H1975) or without (NCI-H3255) the...
secondary T790M mutation. One subtle difference between these cell lines was in the modulation of Hsp90 levels in response to geldanamycin. Levels were stable to very slightly decreased in NCI-H1650 and NCI-H3255 cells and increased in NCI-H1975 cells as well as in cells expressing wild-type EGFR. Both depletion and induction of Hsp90 have been observed in responsive cell lines (34, 35) and may reflect cell line–specific differences in the ability of drug-bound Hsp90 to be ubiquitinated and degraded. Examination of additional NSCLC cell lines will be required to determine whether Hsp90 levels are routinely more persistent in cells harboring the T790M mutation. Nonetheless, other Hsp90 clients were similarly depleted from the mutant cell lines, and all of the mutant EGFRs were detected in complex with the Hsp90 chaperone. Similar immunoprecipitation Western analysis showed at most a very weak interaction between wild-type EGFR and Hsp90 even in cells with EGFR amplification (A-431), consistent with previously published results (19).

As wild-type EGFR is depleted slowly from cells following geldanamycin-mediated Hsp90 inhibition, it is likely that there is transient association of nascent protein with the chaperone, such that the decreased levels seen over time represent compromised appearance of newly synthesized protein. In contrast, similar to HER2 and other oncogenic kinases, mature mutant EGFR likely maintains interaction and dependence on the chaperone, such that

**Figure 5.** Geldanamycin induces apoptosis in NSCLC cell lines harboring EGFR mutation. **A,** Cells (5 × 10^3 per well) were seeded on 96-well plates and grown in the absence or presence of the indicated concentrations of geldanamycin for 72 hours. At 24-hour intervals, dehydrogenase activity was determined using the CCK-8 colorimetric assay. For each cell line, results of a single experiment are shown, in which each determination was done in triplicate. Calu-1, A549, and NCI-H358 express wild-type EGFR. **B,** A549 and NCI-H3255 cells were treated with 1,000 nmol/L geldanamycin for 48 hours and harvested for flow cytometry. A549 cells, representative of EGFR wild-type cell lines, undergo G1-S and G2-M arrest in response to geldanamycin; NCI-H3255 cells, representative of EGFR-mutant cell lines, show a sub-G1 peak following geldanamycin exposure. **C,** EGFR wild-type and mutant cell lines were exposed to 500 nmol/L geldanamycin for 72 hours. Cells were harvested and subjected to a flow cytometry–based TUNEL assay. The percentage of cells showing fluorescein shift in the presence of TdT was used to quantitate apoptosis. DNA fragmentation in this assay is readily detected in the three EGFR-mutant cell lines but only to a small degree in several EGFR wild-type cell lines. Columns, average of three experiments; bars, SE. **D,** EGFR wild-type and mutant cell lines were treated with the indicated concentrations of geldanamycin for 24 hours. Lysates were subjected to Western blotting with an anti-cleaved PARP antibody, confirming apoptosis in response to drug in EGFR-mutant cells.
the Hsp90 inhibition leads to rapid degradation and more complete depletion (19). Therefore, Hsp90 function appears essential to maintain high-level expression of mutant EGFR in NSCLC cells.

As EGFRvIII has also been reported to associate with Hsp90 (22), both types of EGFR mutants, containing alterations in either the kinase or the extracellular domain, associate with Hsp90 to a greater degree than wild-type EGFR. The enhanced association of mutant EGFR parallels recent studies showing that mutant BRAF preferentially interacts with Hsp90 compared with the wild-type protein (17). Similarly, constitutively active v-Src also preferentially interacts with Hsp90 compared with normal cellular Src (36). Why kinase mutation confers greater chaperone association and dependence is unclear. Although it is possible that the altered phosphorylation state of the mutant kinases might contribute to such interactions, both tyrosine-phosphorylated and nonphosphorylated HER2 proteins have been shown to have similar sensitivity to Hsp90 inhibition (19). Alternatively, mutation may result in an inherently less stable structure that requires chaperone machinery not only for proper folding but also for the maintenance of stability and ultimately for protein accumulation. Instability of mutant EGFR may account for the frequent association of somatic mutation with increased gene copy number (37).

Although our work has focused on the interaction of mutant EGFRs with Hsp90, Hsp90 does not interact with client proteins on its own but forms complexes with other proteins, some of which have chaperone-like activity. One of these proteins is Cdc37, which promotes the interaction of Hsp90 with protein kinases, including cdk4 and c-Raf (16, 38). EGFRvIII has been shown to physically interact with Cdc37 and it will be of interest to determine whether the same is true for EGFRs harboring kinase domain mutations (22). Cdc37 is able to function as an oncogene, as mouse mammary tumor virus-Cdc37–expressing mice develop mammary tumors, although with long latency, suggesting a requirement for additional genetic events (39). In this regard, it is possible that Cdc37 and mutant EGFR collaborate in the transformation of the bronchial epithelium.

The precise mechanism by which mutant EGFR is degraded also remains to be elucidated. An E3 ubiquitin ligase may be recruited to the mutant EGFR/Hsp90 complex by geldanamycin. Phosphorylation at Tyr1045, the docking site of the c-Cbl E3 ubiquitin ligase, is similar in wild-type and mutant EGFR proteins (4). However, it is possible that the ubiquitin E3 ligase CHIP is involved in a manner analogous to its geldanamycin-mediated recruitment to HER2/ Hsp90 complexes (40).

Downstream signaling events in EGFR-mutant NSCLC cells appear analogous to those reported in HER2 amplified breast carcinoma cell lines and are similarly disrupted by geldanamycins. For example, among breast cancer cell lines, those expressing high levels of HER2 are among the most sensitive to Hsp90 inhibition, with antiproliferative effects observed at concentrations of geldanamycin derivatives 10– to 100-fold lower than cells without HER2 overexpression (41). In HER2-overexpressing cells, low nanomolar concentrations of ansamycins induce abrupt degradation of HER2, with concomitant loss of ErbB3-associated PI3K activity, and decreased Akt activity, before the loss of Akt protein, which occurs more slowly in response to drug (42, 43). Disruption of this pathway has been shown to result in rapid loss of cyclin D1 expression (42). Cyclin D1 is required for transformation by HER2 (44, 45), so that a critical effector of HER2-mediated oncogenicity is removed. Hsp90 inhibition also leads to apoptosis in these cells, whereas in low HER2-expressing breast cancer cell lines apoptosis is less prominent and occurs later (41).

Similarly, in NSCLC cell lines sensitive to gefitinib, ErbB3 is used to couple EGFR to the PI3K-Akt pathway (27). Thus, the destabilization of mutant EGFR by geldanamycin likely disrupts EGFR–ErbB3 heterodimers, resulting in rapid depletion of phospho-Akt before the more gradual loss in total Akt that occurs in most cell lines in response to Hsp90 inhibition. Compared with EGFR wild-type cell lines, cyclin D1 expression is abruptly compromised after geldanamycin treatment in EGFR-mutant cells. In addition, apoptosis in response to geldanamycin is readily detected in EGFR-mutant cell lines as assessed by both TUNEL assay and PARP cleavage. Further experiments will be required to determine if the depletion of cyclin D1 and the induction of apoptosis are linked or whether other Akt-dependent pathways are primarily responsible for cell death once EGFR is degraded. Nonetheless, the results raise the possibility that cyclin D1 is critical for transformation mediated by mutant EGFR in a manner similar to that described for HER2. Furthermore, depletion of mutant EGFR and phospho-Akt by geldanamycin may sensitize NSCLC cells to chemotherapy agents, as has been described in breast cancer cells (46) and HER2-overexpressing lung cancer cells (47).

Just as HER2-overexpressing breast cancer cell lines are more sensitive to ansamycins compared with cells expressing low levels of HER2, EGFR-mutant NSCLC cell lines are more susceptible to apoptosis than those expressing wild-type EGFR. The EGFR wild-type cell lines surveyed here undergo cell cycle arrest following geldanamycin; arrest at the G1–S boundary correlates in most cases with loss of cdk4 and cdk6 from these cells followed by induction of Cip/Kip proteins (14). In contrast, the rapid depletion of Akt activity in EGFR-mutant cells compromises their viability.

Nonetheless, our results do not necessarily predict that EGFR mutation will fully govern the response to geldanamycins in NSCLC. Some EGFR wild-type NSCLC may undergo apoptosis in response to ansamycins. For example, cdk4 amplification (48) or BRAF mutation (49, 50) have been shown in ~4% and 3% of NSCLCs, respectively, which may define alternative subsets dependent on these kinases for viability and hence more sensitive to Hsp90 inhibition. Conversely, BRAF mutation, present with highest frequency in melaninoma compared with other cancer cell types (51), has not correlated with sensitivity of melanoma cell lines to geldanamycins; in fact, the most sensitive melanoma cell line in a recently reported panel expressed HER2 (35).

Several geldanamycin derivatives are in early-phase human testing, including 17-allylamino-17-demethoxygeldanamycin (17AAG; ref. 52) and 17DMAG. 17AAG is overall well tolerated (53), without the hepatic toxicity that precluded clinical development of geldanamycin itself (54). 17DMAG is water soluble, with excellent bioavailability and tissue distribution shown in preclinical models (55). We have confirmed that 17DMAG causes destabilization of mutant EGFR similar to that induced by parental geldanamycin. The potent disruption of mutant EGFR expression and pharmacologic inhibition of Akt activation by these agents make them attractive for clinical trial in EGFR-mutant–driven NSCLCs.

Acknowledgments


Grant support: Career Development Award as part of the Dana-Farber/Harvard Cancer Center Specialized Programs of Research Excellence in Lung Cancer, NIH grant P20 CA90578 (T. Shimamura).

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We thank Kathryn Folz-Donahue and Laura Prickett (Dana-Farber Flow Cytometry Core) for technical assistance.
References


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