Hsp90 Inhibition Overcomes HGF-Triggering Resistance to EGFR-TKIs in EGFR-Mutant Lung Cancer by Decreasing Client Protein Expression and Angiogenesis

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Introduction: The three major clinically relevant mechanisms of acquired resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) in EGFR mutant lung cancer are a second mutation in the EGFR gene (T790M), Met amplification, and increased expression of hepatocyte growth factor (HGF). Heat shock protein90 (Hsp90) is a 90 kDa molecular chaperone for proteins that include EGFR, Met, and echinoderm microtubule-associated protein-like-4-the anaplastic lymphoma kinase. Here, we determined whether inhibition of Hsp90 could overcome HGF-triggered EGFR-TKI resistance in EGFR mutant lung cancer cells.

Methods: The effects of the Hsp90 inhibitor 17-demethoxygeldanamycin (17-DMAG) on the growth of lung cancer cells resistant to the EGFR-TKI were examined in the presence and absence of HGF, and in cells transfected with the HGF gene in vitro and vivo.

Results: The EGFR-TKI erlotinib did not inhibit the growth of HGF-gene transfected Ma-1 (Ma-1/HGF) cells and H1975 cells, containing the EGFR L858R and T790M mutations, respectively. Erlotinib also did not inhibit the growth of PC-9 and Ma-1 cells, with deletions in EGFR exon19, in the presence of HGF. However, 17-DMAG induced apoptosis and markedly inhibited the growth of these cell lines, even in the presence of HGF. This inhibition by 17-DMAG was associated with decreased expression of EGFR and Met in tumor cells. An in vivo model of HGF-triggered erlotinib-resistance, which used Ma-1/HGF cells, showed that 17-DMAG markedly suppressed tumor growth by decreasing angiogenesis and increasing apoptosis.

Conclusions: Hsp90 inhibitors may overcome HGF-triggered resistance to EGFR-TKIs and may result in more successful treatment of patients with EGFR-mutant lung cancers.

Key Words: HGF, EGFR-TKI resistance, Hsp90, Lung cancer, EGFR mutation.

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inhibition on HGF-induced resistance to EGFR-TKIs in EGFR-mutant lung cancer cell lines in vitro and in vivo.

MATERIALS AND METHODS

Cell Cultures and Reagents

The EGFR-mutant human lung adenocarcinoma cell lines PC-9 (del E746_A750) and HCC827, with deletions in EGFR exon 19, were purchased from Immuno-Biological Laboratories Co. (Takasaki, Gunma, Japan) and the American Type Culture Collection (Manassas, VA), respectively. Ma-1 cells with an EGFR exon 19 deletion, HCC827/ER cells with deletions in EGFR exon 19 and Met amplification, and H1975 cells with the L858R/T790M double mutation, were kindly provided by Dr. E. Shimizu (Tottori University, Tottori, Japan), Dr. T. Mitsudomi (Aichi Cancer Center Hospital, Nagoya, Japan), and Dr. Y. Sekido (Aichi Cancer Center Research Institute, Nagoya, Japan), respectively. Human HGF-gene transfectant (Ma-1/HGF) and vector control (Ma-1/Vec) cells were established as described. All of these cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. All cell lines were passaged for less than 3 months before renewal from frozen, early-passage stocks. Cells were regularly screened for mycoplasma, using MycoAlert Mycoplasma Detection Kits (Lonza, Rockland, ME). Erlotinib, CL-387,785, and 17-DMAG were obtained from Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan), Calbiochem (San Diego, CA), and Selleck Chemicals (Houston, TX), respectively. Human recombinant HGF was prepared as described.

HGF Production in Cell Culture Supernatants

Cells (2 × 10^6) were cultured in 2 ml of RPMI 1640 or Dulbecco’s modified eagle medium with 10% FBS for 24 hours, washed with phosphate-buffered saline (PBS), and incubated for 48 hours in RPMI 1640 with 10% FBS. The culture media were harvested and centrifuged, and the supernatants were stored at −70°C until analysis. HGF was measured by enzyme-linked immunosorbent assay (Immunis HGF EIA; B-Bridge International, Mountain View, CA; limit of detection, 0.1 ng/ml) according to the manufacturer’s instructions. All samples were assayed in triplicate. Color intensity was measured at 450 nm with a spectrophotometric plate reader. Growth factor concentrations were determined by comparison with standard curves.

Cell Growth Assay

Cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) dye reduction method. Tumor cells, plated at 2 × 10^4/100 µl RPMI 1640 plus 10% FBS per well in 96-well plates, were incubated for 24 hours; erlotinib, CL-387,785, 17-DMAG, and/or HGF were added to each well, and incubation was continued for an additional 72 hours. Cell growth was measured with MTT solution (2 mg/ml; Sigma, St. Louis, MO), as described. Each experiment was performed at least three times, each with triplicate samples.

Antibodies and Western Blotting

Protein aliquots of 25 µg each were resolved by sodium dodecyl sulfate polyacrylamide gel (Bio-Rad, Hercules, CA) electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad). After washing four times, the membranes were incubated with Blocking One (Nacalai Tesque, Inc., Kyoto, Japan) for 1 hour at room temperature and overnight at 4°C with primary antibodies to -actin (13E5), MET (25H2), phospho-Met (anti-p-Met, Y1234/Y1235; 3D7), p-EGFR (Y1068), Akt or p-Akt (S473) (Cell Signaling Technology, Beverly, MA), human EGFR (1 µg/ml), human/mouse/rat ERK1/ERK2 (0.2 µg/ml), and p-ERK1/ERK2 (T202/Y204; 0.1 µg/ml) (R&D Systems, Minneapolis, MN). After three washes, the membranes were incubated for 1 hour at room temperature with species-specific horseradish peroxidase–conjugated secondary antibodies. Immunoreactive bands were visualized with SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, Rockford, IL), an enhanced chemiluminescent substrate. Each experiment was performed at least three times independently.

Cell Apoptosis Assay

Cell apoptosis induced by erlotinib and 17-DMAG was measured with an Annexin V-fluorescein isothiocyanate Apoptosis Detection Kit I (BD Biosciences, San Jose, CA) in accordance with the manufacturer’s directions. Cells were analyzed on a FACSCalibur flow cytometer with CellQuest Software (Becton Dickinson, Franklin Lakes, NJ).

Xenograft Studies in Severe Combined Immunodeficiency Mice

Suspensions of Ma-1/Vec and Ma-1/HGF cells (5 × 10^6) were injected subcutaneously into the backs of 5-week-old female severe combined immunodeficiency mice (Clea, Tokyo, Japan). After 7 days, the mice were randomized to (1) no treatment (control group), (2) oral erlotinib (20 mg/kg/day), (3) intraperitoneal 17-DMAG (10 mg/kg/day in water), or (4) oral erlotinib plus intraperitoneal 17-DMAG. Tumor size was measured twice per week, and tumor volume was calculated, in mm³, as width² × length/2. All animal experiments complied with the guidelines for the Institute for Experimental Animals, Advanced Science Research Center, Kanazawa University, Kanazawa, Japan (approval no. AP-081088).

Histological Analyses

Apoptotic cells were detected by terminal deoxynucleotidyl transferase (TUNEL)–mediated nick end labeling staining, using the DeadEnd Fluorometric TUNEL system (Promega, Madison, WI). Briefly, formalin-fixed, paraffin-embedded sections (4 µm thick) were deparaffinized and tissues were permeabilized with protease K solution. The samples were equilibrated, and DNA strand breaks were labeled with fluorescein-12-2-deoxy-uridine-5-triphosphate by adding a nucleotide mixture and TUNEL. The reaction was stopped by the addition of saline sodium citrate, and the localized green fluorescence of apoptotic cells was detected by fluorescence microscopy (>400). Proliferating cells were detected by incubating tissue sections with Ki-67 antibody (Clone MIB-1; DAKO Corp., Glostrup, Denmark). Antigen was retrieved by microwaving tissue sections in 10 mM citrate buffer (pH 6.0). After incubation with secondary antibody and treatment with the Vectastain ABC Kit (Vector Laboratories,
Burlingame, CA), peroxidase activity was visualized using the diamino benzidine (DAB) reaction. To analyze microvessel density, frozen sections (5-µm thick) of xenograft tumors were fixed with cold acetone and washed with PBS. After blocking endogenous peroxidase activity with 3% aqueous H₂O₂ solution for 10 minutes, the sections were incubated with 5% normal horse serum, washed, and incubated overnight at 4°C with antimouse-CD31 (clone MEC13.3, BD Bioscience) antibody. After washing with PBS, the sections were incubated with peroxidase conjugated antirat IgG (Cell Signaling Technology) for 40 minutes. Peroxidase activity was visualized with DAB reactions. The sections were counterstained with hematoxylin.

EGFR was assessed immunohistochemically, using the EGFR pharmDx kit (DAKO), as recommended by the manufacturer. To detect Met protein, formalin-fixed paraffin-embedded tissue sections were incubated with a 1:100 dilution of polyclonal rabbit anti-MET antibody overnight at 4°C after microwave antigen retrieval in 0.01 M citrate buffer (pH 6.0). After incubation with secondary antibody and treatment with the Vectastain ABC Kit), peroxidase activity was visualized using DAB reactions.

Quantification of Immunohistochemistry and Immunofluorescence

The five areas containing the highest numbers of stained cells within a section were selected for histologic quantitation by light or fluorescent microscopy at 400-fold magnification. All results were independently evaluated by two investigators (H.K. and T.N.).

Statistical Analysis

Between-group comparisons were assessed by two-tailed Student’s t tests. All analyses were performed using GraphPad Software (GraphPad Software, Inc., La Jolla, CA). A p value < 0.001 was considered statistically significant.²³

RESULTS

17-DMAG Inhibits Growth of EGFR-Mutant Lung Cancer Cells in the Presence of HGF

PC-9, HCC827, and Ma-1 are human lung adenocarcinoma cell lines with deletions of exon 19 of EGFR, resulting in constitutive activation of this gene. These three cell lines were sensitive to erlotinib, whereas HGF induced erlotinib resistance (Fig. 1A, B, D). The Hsp90 inhibitor 17-DMAG alone markedly inhibited the growth of these three cell lines, with an IC₅₀ (half maximal inhibitory concentration) lower than that of erlotinib in PC-9 (0.01 µmol/l versus 0.03 µmol/l) and Ma-1 (0.01 µmol/l versus 0.03 µmol/l) cells and a similar IC₅₀ in HCC827 (0.01 µmol/l) cells. Under these experimental conditions, HGF did

**FIGURE 1.** 17-DMAG suppresses the in vitro growth of EGFR mutant lung cancer cells in the presence of HGF. Tumor cells were continuously treated with increasing concentrations of EGFR-TKI, erlotinib (PC-9, HCC827, Ma-1, Ma-1/Veg, and Ma-1/HGF), CL-387,785 (H1975), or 17-DMAG, with or without HGF (20 ng/ml), and cell growth was determined after 72 hours by MTT assay. Data shown are representative of five independent experiments. Error bars indicate SD of triplicate cultures. EGFR, epidermal growth factor receptor; HGF, hepatocyte growth factor; TKI, tyrosine kinase inhibitor.
not decrease the sensitivity of the three cell lines to 17-DMAG. In parallel experiments, we used H1975, a human lung adenocarcinoma cell line with both an exon 20 T790M gatekeeper mutation and an exon 21 L858R mutation in \( \text{EGFR} \). Although these cells were resistant to erlotinib (data not shown), they were sensitive to an irreversible EGFR-TKI CL-387,785 \(^{24} \) as described (Fig. 1C). We found that 17-DMAG alone inhibited the growth of H1975 cells, with an IC\(_{50}\) much lower than that of CL-387,785 (0.03 µmol/l versus 0.5 µmol/l); and also inhibited the growth of HCC827ER cells, with a deletion of exon 19 of \( \text{EGFR} \) and \( \text{Met} \) gene amplification, with an IC\(_{50}\) of 0.03 µmol/l (Supplemental Figure 1, Supplemental Digital Content, http://links.lww.com/JTO/A267). HGF did not decrease the sensitivity of H1975 and HCC827ER cells to 17-DMAG. Incubation of PC-9, H1975, and HCC827ER cells, harboring mutant \( \text{EGFR} \), with the combination of 17-DMAG plus EGFR-TKI showed that 17-DMAG did not sensitize these cells to EGFR-TKI treatment in vitro (Supplemental Figures 1 and 2, Supplemental Digital Content, http://links.lww.com/JTO/A267). Taken together, these results suggest that 17-DMAG may overcome HGF-induced resistance to reversible and irreversible EGFR TKIs in lung cancer cells containing an \( \text{EGFR} \) activating and gatekeeper mutation, respectively.

Our previous study demonstrated that, in patients with non–small-cell lung cancer, HGF is detected primarily in cancer cells with acquired resistance to EGFR-TKIs, suggesting that the production of HGF by these cells occurs via an autocrine mechanism. To further explore the effect of 17-DMAG on autocrine HGF, we generated stable HGF-gene transfectants in Ma-1 cells (Ma-1/HGF); as a control, we generated Ma-1/Vec cells transfected with vector alone. Ma-1/HGF cells secreted high concentrations of HGF (27.8 ± 0.9 ng/ml), whereas the concentrations of HGF secreted by Ma-1 and Ma-1/Vec cells were under the limit of detection (Supplemental Figure 3, Supplemental Digital Content, http://links.lww.com/JTO/A267). In addition, Ma-1/HGF cells became resistant to erlotinib. We found that 17-DMAG inhibited the growth of both Ma-1/Vec and Ma-1/HGF cells, with IC\(_{50}\)s of 0.01 µmol/l for both (Fig. 1E and 1F), whereas the combination of 17-DMAG plus erlotinib did not inhibit the growth of Ma-1/HGF cells, indicating that 17-DMAG did not sensitize these cells to erlotinib in vitro (Supplemental Figure 2, Supplemental Digital Content, http://links.lww.com/JTO/A267). These findings indicated that 17-DMAG circumvented resistance to HGF via both autocrine and paracrine mechanisms.

**17-DMAG Reduces Met Protein Expression and Inhibits Downstream Pathways Even in the Presence of HGF**

To explore the molecular mechanism by which 17-DMAG inhibited cell growth, even in the presence of HGF, we examined the protein expression and phosphorylation status of Met, EGFR, and their downstream molecules (PI3K/Akt and ERK1/2) in PC-9 and Ma-1 cells by Western blotting (Fig. 2). PC-9 and Ma-1 cells expressed

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**FIGURE 2.** 17-DMAG suppresses EGF and Met protein expression and phosphorylation even in the presence of HGF. PC-9, Ma-1, Ma-1/Vec, and Ma-1/HGF tumor cells were treated with or without erlotinib (0.3 µmol/l) or 17-DMAG (0.3 µmol/l) for 24 hours, and then stimulated with or without HGF (20 ng/ml) for 10 minutes. The resultant cells were lysed, and the indicated proteins were detected by immunoblotting. EGF, epidermal growth factor; HGF, hepatocyte growth factor.
EGFR and Met proteins, both of which were phosphorylated, as were the downstream molecules Akt and ERK1/2. Although HGF alone did not affect the phosphorylation of EGFR, it stimulated the phosphorylation of Met, thereby activating Akt and ERK1/2. In the absence of HGF, erlotinib inhibited the phosphorylation of EGFR, but not of Met, thereby inhibiting the phosphorylation of Akt and ERK1/2. In the absence of HGF, erlotinib inhibited the phosphorylation of EGFR, but not of Met, thereby inhibiting the phosphorylation of Akt and ERK1/2. In the presence of HGF, erlotinib failed to inhibit the phosphorylation of Met, Akt, and ERK1/2, although it inhibited EGFR phosphorylation. 17-DMAG decreased the expression of EGFR and Met proteins and inhibited their phosphorylation and the phosphorylation of Akt and ERK1/2, irrespective of the presence of HGF. Similar results were observed in Ma-1/HGF cells. These results suggested that 17-DMAG overcomes resistance to HGF, predominantly by inhibiting the expression of EGFR and Met proteins, and then inhibits the expression of the downstream proteins Akt and ERK1/2.

17-DMAG Induces Apoptosis of EGFR-Mutant Lung Cancer Cells Even in the Presence of HGF

Flow-cytometry analyses with Annexin V further confirmed that erlotinib induced apoptosis of Ma-1/Vec cells in the absence, but not the presence, of HGF (Supplemental Figure 4, Supplemental Digital Content, http://links.lww.com/JTO/A267). In contrast, 17-DMAG induced apoptosis in both the presence and absence of HGF. Moreover, 17-DMAG, but not erlotinib, induced apoptosis of Ma-1/HGF cells. These findings indicated that 17-DMAG induces apoptosis and reduces cell growth even in the presence of HGF.

17-DMAG Overcomes HGF-Induced Erlotinib Resistance In Vivo

We next evaluated whether 17-DMAG could overcome HGF-induced resistance to erlotinib in vivo. Oral administration of erlotinib and/or 17-DMAG markedly inhibited the growth of Ma-1/Vectumors (Fig. 3A and 3C). In contrast,
erlotinib failed to inhibit the growth of Ma-1/HGF-tumors, indicating that HGF induced resistance to erlotinib in vivo. Under these experimental conditions, 17-DMAG markedly suppressed the growth of Ma-1/HGF tumors, whereas the combination of 17-DMAG plus erlotinib did not sensitize to erlotinib (Supplemental Figure 5, Supplemental Digital Content, http://links.lww.com/JTO/A267). These results indicated that 17-DMAG may overcome HGF-induced resistance to EGFR-TKIs in vivo (Fig. 3B and 3C).

**17-DMAG Inhibits EGFR and Met Expression and Angiogenesis In Vivo**

We finally assessed angiogenesis and expression of EGFR and Met proteins in Ma-1/HGF-tumors treated with or without erlotinib or 17-DMAG. Control tumor cells expressed both EGFR and Met. Although treatment with erlotinib did not affect their expression of EGFR or Met, treatment with 17-DMAG inhibited their expression of EGFR and Met, indicating the mode of action of 17-DMAG. Moreover, treatment with 17-DMAG, but not erlotinib, increased the number of apoptotic cells. However, the numbers of proliferating control, erlotinib-treated, and 17-DMAG-treated cells were similar (Fig. 4, Fig. 5A and 5B, Supplemental Figure 6, Supplemental Digital Content, http://links.lww.com/JTO/A267). Moreover, 17-DMAG treatment inhibited the angiogenesis of Ma-1/HGF tumors (Fig. 5C, Supplemental Figure 7, Supplemental Digital Content, http://links.lww.com/JTO/A267). These results suggested that 17-DMAG inhibited tumor growth by inducing tumor-cell apoptosis, presumably by affecting the expression of EGFR and Met and by decreasing angiogenesis.

**DISCUSSION**

We have shown here that inhibition of Hsp90 by 17-DMAG could reduce the levels of EGFR and Met proteins and angiogenesis even in the presence of HGF, thus inhibiting the growth of EGFR-mutant lung cancer cells in vitro and in vivo. These findings suggest that HGF-induced EGFR-TKI-resistance in EGFR-mutant lung cancer could be overcome by Hsp90 inhibition. Hsp90 is involved in stabilizing various oncogene products, including mutant EGFR and echinoderm microtubule-associated proetin-like-4-the anaplastic lymphoma kinase (EML4-ALK) fusion protein. Hsp90 stabilizes various types of mutant EGFR, including proteins with exon 19 deletions and the T790M and L858R mutations, and amplified Met. We found that Hsp90 is also involved in the expression of Met stimulated by HGF, suggesting that inhibition of Hsp90 may overcome all three major mechanisms of resistance to EGFR-TKIs in EGFR-mutant lung cancers, including the T790M gatekeeper mutation, Met amplification, and increased expression of HGF. Furthermore, Hsp90 inhibitors are likely effective in EML4-ALK lung cancers, overcoming acquired resistance to ALK inhibitors caused by ALK gene amplification and the L1196M gatekeeper mutation in ALK. Thus, Hsp90 may be an ideal target in patients with lung cancers containing oncogene alterations, such as EGFR.

![FIGURE 4](image-url) 17-DMAG inhibits EGFR and Met expression in vivo. Ma-1/Vec or Ma-1/HGF cells (5 x 10⁶ each) were inoculated subcutaneously into SCID mice on day 0. Mice received oral erlotinib (20 mg/kg/day) or intraperitoneal 17-DMAG (10 mg/kg/day), starting on day 7. Two hours after treatment on day 11, the mice were sacrificed, and their tumors were harvested and examined histologically. Sections were stained with DAPI (nuclear stain), TUNEL (FITC), EGFR (IHC), and Met (IHC), respectively. Bars indicate 50 μm. 17-DMAG, 17-demethoxygeldanamycin; EGFR, epidermal growth factor; HGF, hepatocyte growth factor; SCID, severe combined immunodeficiency; DAPI, 4',6-diamidino-2-phenylindole; TUNEL, terminal deoxynucleotidyl transferase; FITC, fluorescein isothiocyanate; EGFR, epidermal growth factor receptor; IHC, immunohistochemistry.
Moreover, transient but intensive inhibition of PI3K/Akt by PI3K inhibitors and gefitinib successfully overcame HGF-induced EGFR-TKI resistance in vitro and in vivo.\textsuperscript{33} Moreover, transient but intensive inhibition of PI3K/Akt by PI3K inhibitors and gefitinib successfully overcame HGF-induced EGFR-TKI resistance in vitro and in vivo.\textsuperscript{33} The feasibility of these strategies, however, should be evaluated carefully in clinical trials. Hsp90 inhibition may have an advantage, however, because inhibition by a single agent may be sufficient. Recently reported results of early-phase clinical trials have shown the feasibility of several Hsp90 inhibitors.\textsuperscript{28,34,35}

HGF is a mediator that regulates multiple biological functions, including cell motility and invasion.\textsuperscript{36} Moreover, HGF plays an important role in angiogenesis by cooperating with vascular endothelial growth factor.\textsuperscript{37} We previously reported that HGF stimulated vascular endothelial growth factor production by EGFR-mutant lung cancer cells, thereby facilitating angiogenesis and tumor growth in xenograft models. Tumors produced by HGF-gene transfected (Ma-1/HGF) cells were more vascularized and grew faster than those produced by control (Ma-1/Vec) cells. Moreover, 17-DMAG treatment inhibited the angiogenesis of Ma-1/HGF, but not Ma-1/Vec tumors, suggesting that 17-DMAG may overcome HGF-induced angiogenesis. Thus, HGF inhibition would suppress angiogenesis, thereby inhibiting tumor progression. Therefore, in HGF-triggered, EGFR-TKI-resistant lung cancers, Hsp90 inhibitors may control tumor growth not only by decreasing client proteins, EGFR and Met, but also by inhibiting HGF-induced angiogenesis.

Our findings, however, indicate that Hsp90 inhibitors have limitations. Although our in vivo experiments showed that 17-DMAG reduced tumor growth significantly, it failed to shrink tumors produced by Ma-1/Vec and Ma-1/HGF cells. These results are in agreement with previous findings, showing that treatment with the Hsp90 inhibitor, IPI-504, markedly suppressed the growth of H3122 tumor cells containing the EML4-ALK fusion gene, but that these tumors regrew during treatment.\textsuperscript{29} Further experiments are warranted to clarify this mechanism; such experiments are now ongoing in our laboratory.

In conclusion, we demonstrated that Hsp90 inhibition may overcome HGF-triggered resistance to EGFR-TKIs in EGFR-mutant lung cancer. Several early-phase clinical trials have shown the efficacy and feasibility of Hsp90 inhibitors in lung cancer patients. Further clinical development of Hsp90 inhibitors is warranted for EGFR-mutant lung cancer patients. Further clinical development of Hsp90 inhibitors may control tumor growth not only by decreasing client proteins, EGFR, and Met, but also by inhibiting HGF-induced angiogenesis.

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