**USP8 Is a Novel Target for Overcoming Gefitinib Resistance in Lung Cancer**

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**Abstract**

**Purpose:** Common treatment modalities for non–small cell lung cancer (NSCLC) involve the EGF receptor-tyrosine kinase inhibitors (EGFR-TKIs) like gefitinib and erlotinib. However, the vast majority of treated patients acquire resistance to EGFR-TKIs, due, in large part, to secondary mutations in *EGFR* or amplification of the *MET* gene. Our purpose was to test ubiquitin-specific peptidase 8 (USP8) as a potential therapeutic target for gefitinib-resistant and -sensitive non–small cell lung cancer (NSCLC).

**Experimental Design:** Testing the effect of knockdown of USP8 and use of a synthetic USP8 inhibitor to selectively kill gefitinib-resistant (or -sensitive) NSCLCs with little effect on normal cells in cell culture and a xenograft mouse model.

**Results:** Knockdown of ubiquitin-specific peptidase 8 (USP8) selectively kills gefitinib-resistant NSCLCs while having little toxicity toward normal cells. Genetic silencing of USP8 led to the downregulation of several receptor tyrosine kinases (RTK) including EGFR, ERBB2, ERBB3, and MET. We also determined that a synthetic USP8 inhibitor markedly decreased the viability of gefitinib-resistant and -sensitive NSCLC cells by decreasing RTK expression while having no effect on normal cells. Moreover, treatment with a USP8 inhibitor led to significant reductions in tumor size in a mouse xenograft model using gefitinib-resistant and -sensitive NSCLC cells.

**Conclusions:** Our results show for the first time that the inhibition of USP8 activity or reduction in USP8 expression can selectively kill NSCLC cells. We propose USP8 as a potential therapeutic target for gefitinib-resistant and -sensitive NSCLC cells. *Clin Cancer Res; 19(14); 3894–904. ©2013 AACR.*

**Introduction**

Lung cancer remains the leading cause of cancer-related deaths worldwide. Non–small cell lung cancer (NSCLC) is the most common form and accounts for 85% of all cases (1). Pharmacologic inhibitors, including gefitinib and erlotinib, of the EGF receptor (EGFR), have shown notable therapeutic effects in patients with specific forms of NSCLCs (2–4). However, despite a positive initial response to EGFR tyrosine kinase inhibitors (TKI), many patients develop resistance to the drugs after varying periods of time (5). Reports suggest that in approximately 70% of cases, resistance arises from a secondary mutation (T790M) in the *EGFR* and/or amplification of the *MET* gene (6, 7). Therefore, novel treatment strategies to suppress the effects of changes in EGFR and MET are needed to successfully overcome gefitinib and erlotinib resistance in lung cancer therapy.

Various new approaches have been proposed to overcome EGFR-TKI resistance in lung cancer. Some recently developed novel inhibitors can attenuate the activity of EGFR even with a secondary T790M mutation (8–10). Amplification of *MET* accounts for 25% of gefitinib resistance cases in NSCLCs (6). A combination treatment with MET and EGFR inhibitors has also been examined as a means of enhancing the treatment outcome (11–13). In addition, the inhibition of related or downstream signaling pathways of EGFR and MET has also met with some success (14–16).

Deubiquitinating enzymes (DUB) primarily belong to the cysteine protease family and mediate the deconjugation of ubiquitin-tagged substrates (17). Ubiquitin-specific proteases (USP) are a subclass of DUBs with specific targets...
of therapeutic importance (18). Because of their highly specific activity and involvement in several human pathologies including cancer, USPs are rapidly emerging as promising targets for drug design (19, 20).

USP8 (UBPy) was originally reported to be involved in cell growth with expression increasing upon induction by serum (21). More recently, various interesting substrates of USP8 have been identified including Nrdp1 (22), ERBB2 (23), and EGFR (24–26). Because USP8 is involved in EGFR degradation, we hypothesized that it mediated the deconjugation of proteins that would otherwise be tagged for degradation. We show that both silencing and pharmacologic inhibition of USP8 (a DUB relevant to RTKs) lead to dramatic downregulation of EGFR, ERBB2, ERBB3, and MET, and subsequent NSCLC death in vitro and in vivo. Moreover, suppression of USP8 selectively kills gefitinib-resistant and -sensitive NSCLC cells of the type representing patients undergoing therapy with RTK inhibitors. Importantly, USP8 inhibition did not affect viability of normal cells. Our findings highlight the potential advantage of targeting USP8 for overcoming drug resistance in and selective killing of NSCLC.

Translational Relevance

Current therapeutic strategies for non–small cell lung cancer (NSCLC) treatment frequently use pharmacologic inhibitors to suppress oncogenic signaling induced by receptor tyrosine kinases (RTK), including EGF receptor (EGFR) family members. However, this approach is eventually rendered ineffective in the majority of cases, due to selective emergence of drug resistance via receptor mutation and amplification. Deubiquitinating enzymes (DUB) mediate the deconjugation of proteins that would otherwise be tagged for degradation. We show that both silencing and pharmacologic inhibition of USP8 (a DUB relevant to RTKs) lead to dramatic downregulation of EGFR, ERBB2, ERBB3, and MET, and subsequent NSCLC death in vitro and in vivo. Moreover, suppression of USP8 selectively kills gefitinib-resistant and -sensitive NSCLC cells of the type representing patients undergoing therapy with RTK inhibitors. Importantly, USP8 inhibition did not affect viability of normal cells. Our findings highlight the potential advantage of targeting USP8 for overcoming drug resistance in and selective killing of NSCLC.

Materials and Methods

Materials

FBS and antibodies against USP8 and β-actin were purchased from Sigma-Aldrich. Gefitinib and erlotinib were purchased from LC Laboratories. Antibodies to detect phosphorylated ERBB3, ERBB2, EGFR, STAT3, and MET and total ERBB3, MET, STAT3, and Akt were purchased from Cell Signaling Technology. ERBB2, EGFR, extracellular signal–regulated kinase (ERK), and phosphorylated ERK antibodies were purchased from Santa Cruz. The antibody for phosphorylated Akt was purchased from GenScript. The Chemiluminescence Detection Kit was purchased from Amersham Pharmacia Biotech. The Protein Assay Kit was obtained from Bio-Rad Laboratories.

Cell culture

H1975, H1650, H441, H1299, HCC827, and gefitinib-resistant HCC827 (HCC827 GR) human NSCLC cell lines were grown in RPMI supplemented with 10% FBS and penicillin/streptomycin. HCC827GR cells were kindly provided by Dr. Pasi A. Jänne (Harvard Medical School, Boston, MA). CCD-8Lu and WI-38 lung fibroblasts were obtained from the American Type Culture Collection (ATCC) and grown in Eagle's Minimum Essential Medium supplemented with 10% FBS and penicillin/streptomycin. Primary cultured human dermal fibroblasts were kindly provided by Dr. Jin Ho Chung (College of Medicine, Seoul National University, Seoul, Republic of Korea). The NL20 human bronchial epithelial cell line was purchased from ATCC and cultured in Ham’s F12 medium with nonessential amino acids, insulin, EGF, transferrin, hydrocortisone, and 4% FBS. Human bronchial/tracheal epithelial cells (HBTEC) and BronchiaLife B/T Medium Complete Kit were purchased from Lifeline Cell Technology. All cells were maintained as monolayer cultures at 37°C in an incubator with an atmosphere of 5% CO2.

Cell viability assay

Cells were seeded in 96-well plates (1,000–3,000 cells per well depending on the cell type) and incubated overnight before treatment. Cell viability was measured using the CellTiter 96 AQueous MTS Reagent (Promega). For Giemsa staining, briefly, cells were washed with PBS and fixed with formaldehyde and washed again before incubation with Giemsa staining solution (Sigma). After 30 minutes of staining, cells were washed and allowed to dry.

Immunoblotting

After cells were seeded in a 100- or 60-mm dish overnight, the indicated chemicals or siRNAs were administered for the times indicated. Harvested cells were disrupted with cell lysis buffer (Pierce) and the proteins were collected. The protein concentration was determined using a dye-binding protein assay kit (Bio-Rad) as described in the manufacturer’s manual. Protein lysate (20–80 µg) was subjected to SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane. After blotting, the membrane was incubated with a specific primary antibody at 4°C overnight. Protein bands were visualized by a chemiluminescence detection kit after hybridization with an AP-linked secondary antibody.

RNA interference

Cells were grown in 60- or 100-mm dishes and transfected with either a USP8-specific siRNA oligonucleotide
GAPDH: 5'-CTCAGACACCATGGGGAAAGGT-3' (forward), 5'-CTCGAGTGGCAGAGTCTGGG-3' (reverse); for EGFR: 5'-CCACCTGTGCCATCCAAC-3' (forward), 5'-TGTTGGGACAGGCCCTCAAGAC-3' (reverse); for ErbB3: 5'-AAGCTCTACTGAGGTTGTCATA-3' (forward), 5'-TGGCAATGGTGTAAGAGTAGC-3' (reverse); for ErbB2: 5'-AGCCGGCAGGACCCAACT-3' (forward), 5'-TTGTGGGCCAGTACAGTGATT-3' (reverse); and for USP8, primers were purchased from Bioneer P216110.

Animals
All animal procedures were conducted in accordance with animal care guidelines provided by Seoul National University (Seoul, Korea). Male nude mice (6-week-old) were purchased from the Institute of Laboratory Animal Resources at Seoul National University. Animals were acclimated for 1 week before the study and had free access to food and water. The animals were housed in climate-controlled quarters with a 12-hour light/dark cycle.

Xenograft model
H1975 (4 x 10⁶), HCC827GR (5 x 10⁶), and HCC827 (5 x 10⁶) cells in 100 μl of media were mixed with 100 μl of Matrigel (BD Biosciences). Cells were implanted subcutaneously in the hind flank of each mouse. Mice were treated when their tumor volume reached approximately 100 mm³ as measured using calipers and volumes were estimated using the equation V = π/6 (l x h x w). The USP8 inhibitor at 0.2 or 1 mg/kg and gefitinib at 1 or 10 mg/kg were administered intraperitoneally every 5 days a week. Tumor volume was measured weekly, and tumor weight was measured after excision on the final day of the experiment. After all mice were sacrificed, partial tumor tissue was fixed in formalin and embedded in paraffin for immunohistochemical analysis.

Immunohistochemistry
Tumor tissue was prepared for immunohistochemical analysis. Tissue sections (5-μm-thick) from 10% neutral formalin solution–fixed paraffin-embedded tissue were cut on silane-coated glass slides and then deparaffinized with xylene and dehydrated through a graded alcohol bath. The deparaffinized sections were boiled in citric acid buffer (pH 6.0) for phospho-ERBB3 and phospho-EGFR and in EDTA buffer for phospho-ERBB2. The VECTAStain Elite ABC Kit (Vector Labs) was used for further detection and primary antibody was incubated overnight at 4°C. ImmPACT DAB (Vector Labs) was used for staining. Mayer’s hematoxylin (Sigma-Aldrich) was applied as a counterstain.

Deubiquitinating enzyme assay
The activity of USP8 was measured by DUB-Glo Protease Assay (Promega). The USP8 protein was purchased from Abcam. The indicated concentrations of USP8 inhibitor were added to the reaction mixture.
Results

Knockdown of USP8 selectively decreases viability of gefitinib-resistant NSCLC cells

To investigate the effects of USP8 knockdown on viability of gefitinib-resistant NSCLC cells, we transfected siRNAs targeting USP8 (si-USP8) or a scrambled mock control (si-control) into 2 gefitinib-resistant NSCLC cell lines (i.e., H1975 and H1650). Cell viability was assessed by Giemsa staining. H1975 and H1650 transfected with si-USP8 showed a dramatic decrease in cell viability compared with mock-transfected cells, indicating that the suppression of USP8 effectively decreases NSCLC cell viability (Fig. 1A and Supplementary Fig. S1C). In contrast, knockdown of USP8 in normal human bronchial epithelial cells, human lung fibroblasts, and primary dermal fibroblasts had no effect on viability (Fig. 1B and Supplementary Fig. S1A and S1C). Effective knockdown of USP8 was confirmed by Western blotting (Fig. 1C). Gefitinib and erlotinib did not show significant effects on viability of NSCLCs or normal cells (Fig. 1A and B). Also, knockdown of USP8 led to induction of apoptosis in NSCLC cells but not in normal bronchial epithelial cells (Supplementary Fig. S2C).

Knockdown of USP8 inhibits the expression of multiple RTKs in gefitinib-resistant NSCLC cells

Because USP8 was reported to regulate RTKs including EGFR and ERBB2 (23, 25), we explored this relationship using an RTK array to compare the effect of USP8 knockdown on 42 human RTKs in H1975 and H1650 cells. Phosphorylated forms of EGFR, ERBB2, ERBB3, and MET were markedly reduced upon USP8 knockdown compared with mock-transfected cells (Fig. 2A). Western blotting confirmed that knockdown of USP8 not only reduced RTK phosphorylation but also the total levels of EGFR, ERBB2, ERBB3, and MET in H1975 and H1650 cells (Fig. 2B). The mRNA levels of EGFR, ERBB2, ERBB3, and MET did not change (Supplementary Fig. S3).

A potent synthetic USP8 inhibitor, 9-ethylxymino-9H-indeno[1,2-b]pyrazine-2,3-dicarbonitrile, selectively suppresses proliferation of NSCLC cells

When knockdown of USP8 led to selective NSCLC cell death, we hypothesized that targeting USP8 with a small-molecule inhibitor might produce similar effects in NSCLCs. 9-Ethylxymino-9H-indeno[1,2-b]pyrazine-2,3-dicarbonitrile, a recently synthesized inhibitor of USP8 (Fig. 3A; ref. 27), effectively attenuated the deubiquitinating activity of USP8 in vitro (Fig. 3B). We next tested the effect of USP8 inhibition on cell viability using multiple cell lines, including the gefitinib-resistant NSCLC cell lines H1975, H1650, and HCC827GR, as well as the gefitinib-sensitive NSCLC cell line HCC827. The normal bronchial epithelial cell HBTEC and NL20 and lung fibroblast cell lines CCD8-Lu and WI-38 were also subjected to USP8 inhibitor treatment as controls.
representing noncancerous cells. USP8 inhibition markedly reduced cell viability in gefitinib-resistant and -sensitive NSCLC cells but exhibited no observable effects on normal control cells (Fig. 3C). Treatment of the USP8 inhibitor induced apoptosis in NSCLC cells but not in bronchial epithelial cells (Supplementary Fig. S4). To better understand the mechanism of selective killing of NSCLCs, we analyzed the endogenous expression levels of USP8, EGFR, ERBB2, ERBB3, and MET and found that their expression levels were substantially increased in NSCLC cells relative to normal cells (Fig. 3D).

**A USP8 inhibitor reduces RTK expression and downstream signaling in NSCLC cells**

We examined the effect of the USP8 inhibitor or gefitinib on RTK levels and downstream signaling in NSCLC cells. Gefitinib-resistant H1975, H1650, and HCC827GR cells as well as gefitinib-sensitive HCC827 cells were exposed to 1 μmol/L USP8 inhibitor or gefitinib. USP8 inhibitor treatment led to the suppression of phosphorylated and total EGFR, ERBB2, ERBB3, and MET in gefitinib-resistant and -sensitive NSCLC cells (Fig. 4A–D). Gefitinib reduced phosphorylation of EGFR without affecting total EGFR and had minimal effects on the phosphorylation of other RTKs (Fig. 4A–D). The USP8 inhibitor substantially suppressed the phosphorylation of STAT3, Akt, and ERKs, which are downstream targets of RTKs in gefitinib-resistant and -sensitive NSCLC cells (Fig. 4A–D). In contrast, gefitinib treatment inhibited phosphorylation of STAT3, Akt, and ERKs in gefitinib-sensitive HCC827 cells (Fig. 4D), but not in gefitinib-resistant NSCLC cells (Fig. 4A–C).

**Inhibition of USP8 enhances colocalization between ubiquitin and target RTKs**

Because treatment with the USP8 inhibitor reduced RTK expression, and USP8 has been reported to deubiquitinate target proteins (23, 25), we investigated the possible role of the USP8 inhibitor in modulating ubiquitination of RTKs. We analyzed ubiquitin localization in relation to EGFR and ERBB2 (i.e., as representative RTKs) after USP8 inhibitor treatment and found that treatment increased colocalization between ubiquitin and both EGFR (Fig. 5A) and ErbB2 (Fig. 5B). We used fluorescence intensity scanning to confirm that colocalizing ubiquitin and EGFR/ERBB2 fluorescent signals was more spatially distinct in control treatments than with the USP8 inhibitor (Supplementary Fig. S5).
Inhibition of NSCLC tumor growth in a xenograft model by a USP8 inhibitor

To investigate the anti-cancer activity of the USP8 inhibitor (USP8i) \textit{in vivo}, gefitinib-resistant NSCLC cells, H1975 and HCC827GR, and gefitinib-sensitive NSCLC HCC827 cells were subcutaneously transplanted into nude mice. The mice were then treated with USP8i (0.2 or 1 mg/kg) or gefitinib (1 or 10 mg/kg). The USP8 inhibitor significantly suppressed both gefitinib-resistant and -sensitive NSCLC cell tumor growth (Fig. 6A and B). Although gefitinib prevented tumor growth in HCC827 xenografts more effectively than the USP8 inhibitor alone (Fig. 6C), it was less effective in gefitinib-resistant H1975 and HCC827GR tumors compared with the same dose of USP8 inhibitor (Fig. 6A and B). To confirm that inhibition of USP8 prevented RTK activation \textit{in vivo}, we assessed the expression of phosphorylated EGFR, ERBB2, and c-MET in the recovered xenograft tumors. Consistent with our Western blotting data for USP8 inhibitor–treated NSCLC cells, phosphorylated EGFR, ERBB3, and c-MET were markedly downregulated in the presence of the USP8 inhibitor in tumor tissues (Fig. 6D).

Discussion

EGFR-TKIs including gefitinib and erlotinib have a long established reputation for therapeutic efficacy in patients...
Figure 4. Effect of an USP8 inhibitor on cell signaling. Cells were treated or not treated with the USP8 inhibitor or gefitinib (1 μmol/L) for 3 to 6 hours (depending on the cell type) to analyze molecular signaling in (A) H1975, (B) H1650, (C) HCC827/GR, and (D) HCC827 cell lines.
with NSCLCs (2, 3, 28). However, despite initial positive responses to these inhibitors, sustained regression of the disease is invariably hampered by the emergence of drug resistance. Identified mechanisms of resistance include gatekeeper mutations in EGFR and the activation of bypass survival signals mediated through the MET and ERBB3 receptors (6, 7). To overcome acquired resistance to EGFR-TKIs, conventional approaches have focused on suppressing the activity of mutant EGFR, MET, or related signaling molecules through novel inhibitors that can bind the mutant receptor conformations (6, 9, 15, 16). However, such approaches are prone to the possibility of selection for further receptor mutations that bring renewed drug resistance or require multiple drug treatments (29, 30). We propose that selective downregulation of the total levels of oncogenic RTKs including EGFR and MET by manipulating a specific mechanism of endogenous protein regulation could provide more effective control over NSCLC growth than that of sensitive lines. Although gefitinib-resistant HCC827GR cells were more sensitive to USP8 inhibitor treatment. Because gefitinib-sensitive HCC827 cells (6) and thus share the same genetic background, they were more sensitive to USP8 inhibitor treatment. We report that USP8 suppression, by either siRNA or synthetic inhibitor, leads to the multiple oncogenic RTK downregulation of EGFR, ERBB2, ERBB3, and MET while simultaneously inducing significant anticancer effects in both gefitinib-resistant and -sensitive NSCLC cells.

Interestingly, the siRNA-mediated knockdown and pharmacologic inhibition of USP8 showed strong selectivity toward suppression of NSCLC growth but had minimal effects on normal cells. We have also determined that basal expression levels of USP8 and its targets, EGFR, ERBB2, ERBB3, and MET, are elevated in lung cancer cells compared with normal cells. These findings provide insight as to why the suppression of USP8 could be specific for cancer cells while leaving normal cells unharmed because many cancers including NSCLCs are addicted to RTK signaling pathways that promote the proliferation, survival, angiogenesis, and metastasis of cancer cells (38, 39). Activating mutations or overexpression of these RTKs can be frequently observed in a wide range of cancers, and their identification typically results in a poor prognosis (40–42). Given the medical importance of EGFR function in cancer, several small-molecule inhibitors and antibodies have been developed that inhibit EGFR and ERBB2 activity (39). Similarly, MET stimulates proliferation, cell scattering, invasion, and protection from apoptosis and has been reported to exist in human tumors with activating mutations, gene amplification, and transcriptional upregulation (43, 44). Various preclinical and clinical trials are now underway with agents including small-molecule inhibitors and antibodies targeting these receptors to determine whether these concepts can translate into therapeutic application (43).

RTKs such as EGFR, ERBB2, ERBB3, and MET have been reported to crosstalk with each other, and one RTK can drive the activity of others (38, 45). The signaling connections between these RTKs, particularly EGFR and MET, has been identified as a cause of EGFR-TKI resistance, necessitating multiple RTK suppression to achieve adequate therapeutic outcomes (6, 29, 46). Because EGFR and ERBB2 have been identified as targets of USP8-related deubiquitination, we sought to determine whether modulation of USP8 activity could influence endogenous levels of these proteins. We report that USP8 suppression, by either siRNA or synthetic inhibitor, leads to the multiple oncogenic RTK downregulation of EGFR, ERBB2, ERBB3, and MET while simultaneously inducing significant anticancer effects in both gefitinib-resistant and -sensitive NSCLC cells.

Figure 5. Inhibition of USP8 enhances colocalization between ubiquitin and target RTKs. H1650 cells were treated with 1 μmol/L USP8i for 90 minutes and then immunostained to detect (A) EGFR or (B) ErbB2 (green) and ubiquitin (red). The merged image includes DAPI nuclear staining (blue). Enlarged image represents area indicated in the white box. Colocalizing immunofluorescence was translated into a yellow signal using Zeiss LAS software.
Figure 6. Effect of the USP8 inhibitor in a mouse xenograft model. A, representative photographs and weights of H1975 tumors formed in nude mice. Thirteen mice were used for each group and mice were sacrificed 6 weeks after cell injection. B, representative photographs and weights of HCC827GR tumors formed in nude mice. Twelve mice were used for each group and mice were sacrificed 4 weeks after cell injection. C, weights of HCC827 tumors formed in nude mice. Eleven mice were used for each group and mice were sacrificed 6 weeks after cell injection. Tumors were weighed after sacrificing mice on the final day of the study. D, the USP8 inhibitor attenuates phosphorylation of EGFR, ERBB3, and MET in tumor tissues. Tumors were formalin-fixed on the day of excision. Immunohistochemical analysis was conducted as described in Materials and Methods. Staining of phosphorylated EGFR, phosphorylated ERBB3, and phosphorylated c-MET.
more dependent a cell becomes to USP8 targets like EGFR, ERBB2, ERBB3, and MET, the higher the sensitivity could become toward USP8 inhibition is not unreasonable. Our phospho-RTK array data using H1975 and H1650 gefitinib-resistant cell lines revealed that the strongest phosphorylation of RTKs were EGFR, ERBB2, ERBB3, and MET, adding further weight to the idea that these RTKs provide prosurvival mechanisms for these cell lines. Interestingly, while USP8 inhibition and knockdown reduced levels of the phosphorylated insulin receptor, it had no effect on total levels of the protein (Supplementary Fig. S6). This supports the idea that USP8 specifically targets substrate molecules as opposed to having broad effects on multiple RTKs. Notably, the RTK status of a given tumor type might be an important factor when selecting tumors for USP8 therapeutic targeting.

Taken together, our findings show for the first time that inhibition of USP8 downregulates the total protein levels of EGFR, ERBB2, ERBB3, and MET and effectively attenuates related RTK signaling pathways. These observations provide proof-of-principle that targeting USP8 can selectively kill cancer cells without adverse effects on normal cells. More specifically, our data suggest that inhibition of USP8 is an effective method for overcoming gefitinib resistance in NCLC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
9. Engelman JA, Zejnullahu K, Gale CM, Lifshits E, Gonzalez AJ, Shi 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.


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