Inhibition of Ubiquitin-specific Peptidase 8 Suppresses Growth of Gefitinib-resistant Non-small Cell Lung Cancer Cells by Inducing Apoptosis

Chul-Ho Jeong
College of Pharmacy, Keimyung University, Daegu, Korea

Background: Therapeutic approach by treatment with epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) like gefitinib or erlotinib to non-small cell lung cancer (NSCLC) patients has been limited due to emergence of acquired drug resistance. Our study was aimed to investigate whether the inhibition of ubiquitin-specific peptidase 8 (USP8) could be an alternative strategy capable of overcoming acquired resistance to EGFR-TKIs for treatment of NSCLCs.

Methods: The anticancer effect of USP8 inhibitor was determined by testing anchorage-dependent or independent growth of gefitinib-sensitive or resistant NSCLCs. The immunoprecipitation and western blotting were conducted to check molecular interaction and signaling pathway followed by USP8 inhibition.

Results: Inhibition of USP8 induced overall degradation of oncogenic receptor tyrosine kinases including EGFR and Met, leading to a suppression of anchorage-dependent or independent cell growth of gefitinib-sensitive or resistant NSCLCs. Also, treatment with the USP8 inhibitor markedly induced apoptosis in HCC827GR cells. Notably, treatment with the USP8 inhibitor was more effective in suppressing cell growth and inducing apoptosis in gefitinib-resistant HCC827GR cells than that of gefitinib-sensitive HCC827 cells.

Conclusions: Inhibition of USP8 could be an effective strategy for overcoming gefitinib resistance in NSCLCs.

Key Words: USP8, Non-small cell lung cancer, Gefitinib, Apoptosis, Resistance

INTRODUCTION

The epidermal growth factor receptor (EGFR) has been regarded as a relevant target in cancer treatment because aberrant overexpression of EGFR was detected and correlated with tumor growth and poor prognosis of various human cancers.\(^1\,^2\) Non-small cell lung cancers (NSCLCs) which account for 85% of all lung cancers are characterized by multiple mutations in the gene encoding EGFR. Notably, it has been reported that somatic mutations including deletion in exon 19 and the L858R mutation in exon 1 of the EGFR gene confer sensitivity to the EGFR-TKIs in a subset of NSCLC patients.\(^3\,^5\) EGFR tyrosine kinase inhibitors (EGFR-TKIs) such as gefitinib or erlotinib, both of which compete with ATP for binding to the tyrosine kinase pocket of the EGFR, have been developed for patients with NSCLCs or other human cancers.\(^4\,^6\) Despite initial responses of EGFR-TKIs, however, drug resistance emerges from the acquisition of a secondary mutation (T790M) in exon 20 of EGFR in >50% of NSCLC patients.\(^8\,\) Furthermore, the amplification of the gene for the Met, a prominent receptor tyrosine kinase (RTK), has been known to confer a gefitinib-resistance in >20% of NSCLC patients.\(^12\) In this regard, several drugs which including irreversible EGFR-TKIs and mutant EGFR-selective EGFR-TKIs have been investigated to overcome the acquired resistance in NSCLCs.\(^14\) Moreover, combinatorial inhibition using dual EGFR-Met-TKIs has been investigated to overcome resistance induced by Met.\(^15\) However, it is still crucial to find the alternative treatment strategies and drugs capable of overcoming acquired resistance to EGFR-TKIs for...
treatment of NSCLCs.

Most well-known deubiquitinating enzymes (DUBs) are cysteine proteases that serve to reverse protein ubiquitination. Ubiquitin-specific proteases (USPs) comprise the largest class of DUBs which remove ubiquitin from specific protein substrates and allow protein salvage from proteasomal degradation, regulation of protein localization or activation. Due to a key role for deubiquitination in degradation of a variety of proteins which involve in human malignancies, USPs have been considered as good targets for cancer treatment. Ubiquitin-specific peptidases (USP8) was originally identified to enhance cell growth as its expression increases upon serum stimulation in cancer cells. It has been reported that USP8 interacts with a number of clinically relevant cancer targets including Cdc25, ERBB2, Nrdp1, and EGFR. In agreement with this, previous animal study clearly showed that the decreased protein level of several RTKs including EGFR, c-Met, and ErbB3 was observed in USP8 conditional knockout mice, implying crucial role of USP8 in cancers. Moreover, our colleges recently reported that inhibition of USP8 overcomes gefitinib-resistance in human NSCLCs through down-regulating the levels of RTKs, suggesting that USP8 might play a role in NSCLCs by stabilization of several oncogenic RTKs including EGFR, ERBB2, and Met.

In this present study, we investigated the anticancer efficacy of USP8 inhibitor in several human NSCLCs. We demonstrate that treatment of USP8 inhibitor suppresses the anchorage-dependent and independent growth of NSCLCs and induces apoptosis by caspase-dependent manners. Moreover, our data reveals that USP8 inhibitor is potent in gefitinib-resistant HCC827GR harboring Met amplification, suggesting that USP8 inhibitor could be a new therapeutic candidate to overcome resistance to EGFR-TKIs in NSCLCs.

MATERIALS AND METHODS

1. Materials

Antibodies for Met, Akt, phospho-Akt (Ser473), cleaved Caspase-3, cleaved polyadenosine diphosphate ribose polymerase (PARP), and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody specific for EGFR was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). SU 11274 and USP8 antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gefitinib was purchased from LC Laboratories (Woburn, MA, USA). Fetal bovine serum (FBS), streptomycin, and penicillin were obtained from Thermo Fisher Scientific (Rockford, IL, USA). Cocktail and EDTA were purchased from Thermo Fisher Scientific (Rockford, IL, USA).

2. Cell culture

The human NSCLC cell lines H1299, H1650, and HCC827 were maintained in RPMI-1640 with L-glutamine containing 10% FBS and 1% penicillin/streptomycin. Gefitinib-resistant HCC827GR cells were cultured in RPMI-1640 with L-glutamine supplemented with 10% FBS, penicillin/streptomycin, and 1 μM gefitinib. The cells were cultured as monolayer at 37°C with an atmosphere of 5% CO2.

3. Cell proliferation assay

Cell proliferation was determined using the CellTiter96® Aqueous One Solution Reagent (Promega, Madison, WI, USA). HCC827 and HCC827GR cells were plated in 96-well flat-bottomed plate at an appropriate cell number and incubated overnight before treatment. After being incubated with USP8 inhibitor, 20 μL of reagent was added to the wells, and the plate was incubated at 37°C for an additional 1 hour. Absorbance at 492 nm was then read on Tecan Infinite F200 Pro plate reader (Promega), and values were expressed as percent of absorbance from cells incubated in dimethyl sulfoxide alone.

4. Soft agar colony formation assay

For anchorage-independent growth assay, H1299, H1650, HCC827, and HCC827GR cells (8 x 10³ cells/well) were suspended in Basal Medium Eagle (BME) (1 mL with 10% FBS, 0.33% agar) and plated over a layer of solidified bottom agar mixture (BME with 10% FBS, 0.5% agar) with indicated concentrations of USP8 inhibitor. The cultures were maintained at 37°C in a 5% CO2 incubator for 6 to 7 days, and the colonies were counted under light microscope.

5. Immunoprecipitation and Western blot analyses

Immunoprecipitation and Western blot analysis were performed using ice-cold lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP40) and 20 to 30 μg of lysate per lane was separated by SDS-PAGE and followed by transferring to a PVDF membrane (Bio-Rad, Hercules, NJ, USA). The membrane was blocked with 5% skim milk in Tris-buffered saline and tween 20 (TBS-T), and then incubated with the corresponding antibodies. After binding of an species-specific secondary antibody coupled to hors eradish peroxidase, proteins were visualized by SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Waltham, MA, USA) and developed with LAS-3000 (Fuji, Tokyo).
Figure 1. Ubiquitin-specific peptidase 8 (USP8) inhibitor suppresses anchorage-independent growth of H1299 and H1650 cells by downregulation of oncogenic receptor tyrosine kinases. (A) Chemical structure of USP8 inhibitor. (B) Effect of USP8 inhibitor on non-small cell lung cancers (NSCLCs) signaling. H1299 and H1650 cells were treated with the indicated concentrations of USP8 inhibitor for 24 hours to analyze molecular responsiveness. (C) Interaction of epidermal growth factor receptor (EGFR) and USP8 in NSCLCs. Cell lysates from H1299 and H1650 cells were subjected to immunoprecipitation (IP) and western blotting (WB) using the control immunoglobulin G (IgG), EGFR, and USP8 antibodies. (D, E) Colony formation of H1299 and H1650 cells after exposure to the increasing concentration of USP8 inhibitor or gefitinib for 7 days. Random areas were scanned (five areas per well, three wells per set) in colonies grown in soft agar. Error bars represent the mean ± SD. Statistical significance was determined by the Student’s t-test (*P < 0.01).
USP8 inhibitor. 9-ethylxymino9H-indeno(1,2-b)pyrazine-2,3-dicarbonitrile (Fig. 1A).25,26 Our data revealed that treatment with 0.25 to 0.5 μM USP8 inhibitor effectively downregulated the expression levels of EGFR, Met, and Akt in gefitinib-sensitive H1650 cells, whereas did not affect in gefitinib-resistant H1299 cells (Fig. 1B). However, treatment with the higher concentration of USP8 inhibitor (5 to 10 μM) showed a robust downregulation of total levels of EGFR, Met, and Akt proteins in H1299 cells (data not shown), suggesting the differential potency of USP8 inhibitor in two cells. Because USP8 has been reported to regulate a number of cancer targets including EGFR,20-22 we first explored whether USP8 might interact with EGFR in NSCLCs. H1299 and H1650 cells. By conducting an Immunoprecipitation assay, we found an interaction between endogenous USP8 and EGFR in both cell lines, confirming that EGFR is a USP8's client protein (Fig. 1C). Next, we investigate the anticancer effect of USP8 inhibitor in NSCLCs by performing the anchorage-independent colony formation assay. Expectedly, our data revealed that treatment with the USP8 inhibitor in H1299 cells resulted in no effect in colony number up to 1 μM, whereas significant decrease in colony number was observed at a concentration of 5 to 10 μM USP8 inhibitor (Fig. 1D). Also, treatment with the USP8 inhibitor in H1650 cells markedly decreased the soft-agar formation in a dose-dependent manner (Fig. 1E). Moreover, a more potent growth inhibitory effect was observed in USP8 inhibitor treated cells compared to gefitinib-treated cells, implying that USP8 inhibitor might have a potential therapeutic efficacy.

**Figure 2.** Ubiquitin-specific peptidase (USP8) inhibitor suppresses anchorage-independent and dependent growth of gefitinib-sensitive HCC827 and gefitinib-resistant HCC827GR cells. (A) Whole cell lysates were assayed by western blot analysis using antibodies against epidermal growth factor receptor (EGFR), Met, and USP8. β-actin was used as a loading control. (B) Colony formation of HCC827 and HCC827GR cells after exposure to the increasing concentration of USP8 inhibitor for 7 days. Random areas were scanned (five areas per well, three wells per set) in colonies grown in soft agar. Error bars represent the mean ± SD. Statistical significance was determined by the Student’s t-test (*P < 0.01). (C) Gefitinib-sensitive HCC827 or resistant HCC827GR cells were treated with various concentrations of indicated drugs for 3 days and cell proliferation was determined using the MTS assay. Error bars represent the mean ± SD. Statistical significance was determined by the Student’s t-test (*P < 0.01).
2. Ubiquitin-specific peptidase 8 inhibitor overcomes gefitinib-resistant non-small cell lung cancer growth

Gefitinib-resistant HCC827GR cells were generated by continuously exposing the HCC827 cells to increasing concentrations of gefitinib as reported.13,27 Our western blot analysis confirmed that gefitinib-resistant HCC827GR cells showed an increased expression level of Met and USP8 protein compared with gefitinib-sensitive HCC827 cells (Fig. 2A). Based on this observation, we next tested the anticancer effect of USP8 inhibitor on gefitinib-sensitive or resistant NSCLCs. The colony formation assay revealed that treatment with the USP8 inhibitor substantially suppressed the anchorage-independent growth of HCC827 and HCC827GR cells in a dose-dependent manner (Fig. 2B). Notably, treatment with the USP8 inhibitor at a 1 to 5 μM concentration showed a more significant decrease in colony number in gefitinib-resistant HCC827GR than HCC827 cells (Fig. 2B). Anti-proliferative effects of USP8 inhibitor, gefitinib, and a Met inhibitor, SU11274, were assessed in these NSCLC cell lines. As a result, treatment with the USP8 inhibitor significantly decreased the proliferation of HCC827 and HCC827GR cells in a dose-dependent manner, whereas an expected marginal effect was observed in gefitinib- or SU11274-treated groups (Fig. 2C). Moreover, anti-proliferative effect of USP8 inhibitor was evidently observed in gefitinib-resistant HCC827GR cells as well, suggesting that USP8 inhibitor has efficacy to overcome acquired resistance to gefitinib in NSCLCs.

3. Ubiquitin-specific peptidase 8 inhibitor potently induces apoptosis in gefitinib-resistant HCC827GR cells

To determine whether anti-proliferative activity of USP8
inhibitor is resulted from the induction of apoptosis, flow-cytometry analysis with annexin V was performed. A flow cytometric analysis with Annexin V showed that treatment with the USP8 inhibitor induced early apoptosis both in gefitinib-sensitive HCC827 cells and gefitinib-resistant HCC827GR cells (Fig. 3A). Interestingly, dose-dependent treatment with 1 to 2.5 μM USP8 inhibitor in HCC827GR cells markedly induced early apoptosis at a level of 29.7% and 40.8%, respectively, but not in cells treated with 1 μM gefitinib. In HCC827 cells, however, gefitinib treatment induced early apoptosis at a level of 33%, whereas a marginal induction level was observed in USP8 inhibitor-treated cells (Fig. 3A). We next compared the total apoptosis level induced by several cancer therapeutic drugs including gefitinib, SU11274, and USP8 inhibitor in these two cell lines. Our fluorescence activated cell sorter (FACS) data revealed that the induction level of total apoptosis was evidently observed in USP8 inhibitor-treated HCC827GR cells (Fig. 3B). Its apoptotic effect was accompanied by the induction of activated caspase-3 and cleaved PARP level (Fig. 3C). Therefore, these findings clearly suggest that treatment with the USP8 inhibitor is sufficient for inducing apoptosis in gefitinib-resistant HCC827GR cells.

**DISCUSSION**

Acquired resistance to EGFR-TKIs inevitably develops in many NSCLC patients by two representative mechanisms - a secondary T790M mutation in EGFR gene and the amplification of Met.9,11,13 To overcome these acquired resistance, treatment with the irreversible EGFR-TKIs targeting T790M-EGFR14,28 or combined treatment of EGFR-TKIs and Met-TKIs have been proposed.15 However, these therapeutic approaches may result in renewed drug resistance because of complexities of cancer cell signaling which activating alternative survival pathways. Therefore, development of new drugs which inhibit diverse oncogenic pathways simultaneously might be more effective. Consistent with this, we recently reported that targeting heat shock protein 90 (HSP90) by WK88-1, a novel geldanamycin derivative, is an alternative treatment strategy overcoming resistance to EGFR-TKIs in NSCLC with Met amplification or T790M mutation in EGFR.20,22 Moreover, we also reported that SB305 might be a good natural compound for use in the treatment of Met-amplified NSCLCs.27

Protein (de)ubiquitination is crucial to the cell cycle regulation and cancer progression. DUBs can reverse protein ubiquitination and are thought to function to promote protein stability. Recent studies have revealed that DUBs emerge as novel anticancer targets because they regulate various oncogenic products and tumor suppressors.31,33 Notably, USP8 has been reported to regulate oncogenic RTKs including EGFR and ERBB2 by deubiquitination,20,22 suggesting the hypothesis that its targeted inhibition could enhance cancer therapies. Consistently, we and our colleges recently proved this hypothesis to show that inhibition of USP8 overcomes gefitinib-resistance in human NSCLCs.25

In this study, we show that USP8 inhibitor has an inhibitory effect on the anchorage-dependent and independent growth of NSCLCs including gefitinib-sensitive and resistant cells. Moreover, inhibition of USP8 by its inhibitor is sufficient for inducing apoptosis in gefitinib-resistant HCC827GR cells, thereby overcomes gefitinib resistance which is a key obstacle to treat NSCLCs. Inhibition of USP8 results in a dramatic decrease of the total protein levels of RTKs including EGFR and Met and subsequently inhibits downstream signaling. Interestingly, treatment with the USP8 inhibitor was more effective in suppressing cell growth and inducing apoptosis in gefitinib-resistant HCC827GR cells than that of gefitinib-sensitive HCC827 cells. Because the HCC827GR cells has been reported to exhibit a high dependency toward Met and EGFR, it might be reasonable that the more dependent a cell becomes to USP8 target proteins, the higher the sensitivity could become toward USP8 inhibition as reported.25 Collectively, our findings suggest that USP8 could be a new therapeutic target for overcoming resistance to EGFR-TKIs in NSCLCs.

**ACKNOWLEDGMENTS**

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012R1A1A1010735) and by College of Pharmacy-specialized Research Fund (from institute for new drug development) of Keimyung University in 2012.

**CONFLICTS OF INTEREST**

No potential conflicts of interest were disclosed.

**REFERENCES**

1. Gullick WJ. Prevalence of aberrant expression of the epidermal growth factor receptor in human cancers. Br Med Bull 1991;47: 87-98.
2. Salomon DS, Brandt R, Cardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. Crit Rev Oncol Hematol 1995;19:183-232.
3. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129-39.

4. Fazek JA, Jänne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497-500.

5. Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria J, et al. EGFR receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci U S A 2004;101:13306-11.

6. Fukuzoka M, Yano S, Giaccone G, Tamura T, Nakagawa K, Douillard JY, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (The IDEAL I Trial) [corrected]. J Clin Oncol 2005;23:2237-46.

7. Shepherd FA, Rodrigues Pereira J, Ciuleanu T, Tan EH, Hirsh V, Thongprasert S, et al. National Cancer Institute of Canada Clinical Trials Group. Erlotinib in previously treated non-small-cell lung cancer. N Engl J Med 2005;353:129-32.

8. Thatcher N, Ch timestep KH, Toms AV, Woo MS, Greulich H, Wong KK, et al. The T790M mutation in EGFR kinase causes drug resistance. J Biol Chem 2007;282:1658-60.

9. Mizzuto E, Iura T, Mukai A, Yoshimori T, Kitamura N, Komada M. Regulation of epidermal growth factor receptor down-regulation by UBPy-mediated deubiquitination at endosomes. Mol Biol Cell 2005;16:5163-74.

10. Nierendorf S, Oksche A, Kissner A, Löbler J, Prinz M, Schorle H, et al. Essential role of ubiquitin-specific protease 8 for receptor tyrosine kinase stability and endocytic trafficking in vivo. Mol Cell Biol 2007;27:5020-39.

11. Byun S, Lee JY, Lee J, Jeong CH, Farrand L, Lim S, et al. USP8 is a novel target for overcoming gefitinib resistance in lung cancer. Clin Cancer Res 2013;19:3904-9.

12. Colombo M, Vallesse S, Peretto I, Jacq X, Rain JC, Colland F, et al. Synthesis and biological evaluation of 9-oxo-9H-indeno[1,2-b]pyrazine-2,3-dicarbonitrile analogues as potential inhibitors of deubiquitinating enzymes. ChemMedChem 2010;5:552-6.

13. Jing WJ, Park B, Jeong GS, Hong SS, Jeong CH, SB365. Pulsatilla saponin D suppresses the growth of gefitinib-resistant NSCLC cells with Met amplification. Oncol Rep 2014;32:2612-8.

14. Zhou W, Erkan D, Chen L, Yun CH, Li D, Capelletti M, et al. Novel mutant-selective EGFR kinase inhibitors against EGFR T790M. Nature 2009;462:1070-4.

15. Hong SY, Jing WJ, Chun KS, Jeong CH. Hsp90 inhibition by WK88-1 potently suppresses the growth of gefitinib-resistant H1975 cells harboring the T790M mutation in EGFR. Oncol Rep 2014;31:2619-24.

16. Jang WJ, Jung SK, Kang JS, Jeong CH. Anti-tumor activity of WK88-1, a novel geldanamycin derivative, in gefitinib-resistant non-small cell lung cancers with Met amplification. Cancer Sci 2014;105:1245-53.

17. Nicholson B, Marblestone JG, Butt TR, Mattern MR. Deubiquitinating enzymes as novel anticancer targets. Future Oncol 2007;3:191-9.

18. Popov N, Wanzel M, Madireddo M, Zhang D, Beijersbergen R, Bernards R, et al. The ubiquitin-specific protease USP8 is required for MYC stability. Nat Cell Biol 2007;9:765-74.

19. Yuan J, Luo K, Zhang L, Cheville JC, Lou Z. USP10 regulates p53 localization and stability by deubiquitinating p53. Cell 2010;140:384-96.