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Role of tyrosine kinase-independent phosphorylation of EGFR with activating mutation in cisplatin-treated lung cancer cells

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ABSTRACT

Epidermal growth factor receptor (EGFR) mutation is one of the hallmarks of cancer progression and resistance to anticancer therapies, particularly non-small cell lung carcinomas (NSCLCs). In contrast to the canonical EGFR activation in which tyrosine residues are engaged, we have demonstrated that the non-canonical pathway is triggered by phosphorylation of serine and threonine residues through p38 and ERK MAPKs, respectively. The purpose of this study is to investigate the role of non-canonical EGFR pathway in resistance mechanism against cisplatin treatment. Wild type and mutated (exon 19 deletion) EGFR-expressing cells responded similarly to cisplatin by showing MAPK-mediated EGFR phosphorylation. It is interesting that internalization mechanism of EGFR was switched from tyrosine kinase-dependent to p38-dependent fashions, which is involved in a survival pathway that counteracts cisplatin treatment. We therefore introduce a potential combinatorial therapy composed of p38 inhibition and cisplatin to block the activation of EGFR, therefore inducing cancer cell death and apoptosis.

1. Introduction

Non-small cell lung carcinomas (NSCLCs), the most common type of lung cancer, are relatively insensitive to chemotherapeutics. Combinatorial chemotherapeutics based on cisplatin (CDDP) has been a widely successful treatment for many types of cancer, including lung cancers [1–4]. Combinatorial therapies based on cisplatin were significantly used for NSCLCs to tackle its resistance. Nevertheless, novel approaches and strategies are required to face newly recognized mechanisms of resistance [5–8].

Epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptor tyrosine kinases (RTKs) [9]. Normally, EGF binds to EGFR to induce preferentially tyrosine autophosphorylation, which causes activation of MAPK and Akt pathways. Mutations in the tyrosine kinase (TK) domain of the EGFR found in high percentage of patients, especially in Asian countries, are associated with the development of cancer due to its constitutive activation [10–12]. Exon 19 deletions or L858R point mutation are the most prevalent of the EGFR mutations in NSCLCs, and cause the enhancement of sensitivity to EGFR TK inhibitors (EGFR-TKIs) [13]. We have demonstrated that, in the presence of cellular stresses, including tumor necrosis factor-α (TNF-α), the activation of p38/ERK induces TK-independent phosphorylation of EGFR at serine/threonine residues [15,16]. This non-canonical EGFR pathway can eventually cause a p38-dependent internalization of EGFR [15–17]. The role of both EGFR and MAPKs on the development of resistance, especially against cisplatin, has been reported [18–24]. Several reports have shown the role of cisplatin in the activation of p38 MAPK, and subsequent non-canonical EGFR pathway [17,25]; however, a clear identification of the phosphorylated residue and its implication was not shown.

In this study, we are investigating the role of cisplatin in activating the non-canonical pathway in human lung adenocarcinoma cells harboring EGFR mutation. We demonstrated the effect of

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blocking the p38-mediated EGFR internalization on enhancing the apoptotic potential of cisplatin.

2. Materials and methods

2.1. Antibodies and reagents

Phospho-specific antibodies against p38 (Thr-180, Tyr-182), ERK (Thr-202, Tyr-204), and EGFR (Tyr-845, 998, 1045 and 1068, Thr-669, and Ser-1046/1047), in addition to PARP-1 and caspase-3 were purchased from Cell Signaling Technology. Antibodies against EGFR (1005), α-Tubulin (B-7) and β-actin (C-11) were obtained from Santa Cruz Biotechnologies. Recombinant human TNF-α and EGF were obtained from R&D System, and HGF was obtained from PeproTech. LY294002, SB203580, U0126 and PD153035 were from Merck Biosciences. 5Z-7-oxozeaenol was a gift from Chugai Pharmaceutical Co. Ltd [26]. Cisplatin (CDDP) and gefitinib were purchased from Wako Pure Chemical and Cayman Chemical, respectively. All chemical inhibitors were dissolved in DMSO and the final concentration of DMSO was less than 0.1%.

2.2. Cell cultures

A549 and PC-9 cells were cultured in RPMI 1640 medium (Life Technologies). HeLa and MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies). Media were supplemented with 10% FBS (ICN Biomedicals, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO_2.

2.3. Immunoblotting

After stimulation, whole-cell lysates were prepared as described previously [27]. Cell lysates were resolved by 7.5%, 10%, or 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to an Immobilon-P nylon membrane (Millipore). The membrane was treated with BlockAce (Dainippon Pharmaceutical, Japan) and probed with primary antibodies as describe above. The antibodies were detected using horseradish peroxidase-conjugated anti-rabbit, anti-mouse, and anti-goat IgG (Dako) and visualized with the enhanced chemi-luminescence system. Some antibody reactions were carried out in Can Get Signal solution (TOYOBO). Representative data from at least 3 independent experiments were selected for the figures.

2.4. Fluorescence microscopy

Cells were seeded on a coverslip (Thermo Fisher) 18 h prior to processing for immunofluorescence. Cells (60% confluent) were fixed with 4% paraformaldehyde for 20 min and then permeabilized with 0.25% Triton X-100 for 5 min. Coverslips were overlaid with anti-EGFR monoclonal antibody (clone CA1; Millipore) and incubated for 2 h and with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (Life Technologies) for 1 hy. After being washed with PBS, coverslips were inverted onto a slide with SlowFade Gold Antifade Reagent with DAPI (Life Technologies). Fluorescence was analyzed by LSM700 confocal microscopy (Zeiss).

2.5. Cell viability assay

Viability of cells was assessed using a WST-1 Cell Counting Kit (Wako Pure Chemical) as described previously [28]. Briefly, cell suspensions in supplemented media were seeded into a 96-well plate ($5–8 \times 10^3$/well), and incubated at 37 °C in a humidified atmosphere of 5% CO_2. After overnight incubation, additional medium containing the inhibitors were added to the wells. After 30 min CDDP were added and the plate were incubated for 12 h. WST-1 solution was added to each well at 2 h before the end of experiment. Absorbance at 450 nm (reference: 620 nm) was measured using a microplate reader. Phase-contrast microscopic images were captured at 10× zoom.

2.6. Statistical analysis

Data are presented as the mean ± S.D. of at least 3 independent experiments. The statistical analysis was performed using JMP software (version 10; SAS Institute). The statistical significance between conditions was determined using Bonferroni correction. $P < 0.01$ was considered to indicate a statistically significant difference.

3. Results

3.1. CDDP induces a non-canonical pathway of wild type EGFR through p38/ERK MAPKs

We and others have reported the role of p38 and ERK in phosphorylation of EGFR at Ser-1046/1047 and Thr-669, respectively, after the stimulation with TNF-α [15–17]. To examine the effect of CDDP on EGFR phosphorylation, we used HeLa cells as well as other EGFR-wild type cell lines, including A549 (lung adenocarcinoma) and MDA-MB-231 (breast cancer) cells. CDDP caused phosphorylation of ERK, p38, and EGFR at non-canonical serine/threonine sites starting from 60 μM, and gradually increased up to 100 μM in HeLa cells (Fig. S1A); therefore, these cells were subjected to 100 μM CDDP to monitor the phosphorylation pattern. Maximum phosphorylation of ERK, p38, and EGFR threonine and serine residues was detected at 6 h (Fig. 1A). CDDP, similar to TNF-α, and in contrast to EGF, triggered non-canonical serine/threonine phosphorylation of EGFR with no tyrosine autophosphorylation (Fig. 1B). These results were further validated using the EGFR tyrosine kinase inhibitor, PD153035, which caused the down-regulation of EGF-induced pY1068, but did not affect the CDDP-induced pT669 and pS1046/1047 (Fig. 1C).To confirm the role of ERK and p38 in the activation of threonine and serine, cells were pretreated with specific MAPK inhibitors. U0126, an inhibitor of MEK-ERK pathway, inhibited CDDP-induced Thr-669 phosphorylation. SB203580, a specific inhibitor of p38, blocked Ser-1046/1047 phosphorylation. This was further confirmed using 5Z-7-oxozeaenol, a TAK1 inhibitor, that inhibited both ERK/Thr-669 and p38/Ser-1046/1047 phosphorylation (Fig. S1B).

3.2. Constitutive phosphorylation of threonine-669 of active mutant EGFR

We managed to extrapolate phosphorylation of mutated EGFR on the basis of our findings on wild type EGFR. PC-9 lung adenocarcinoma cells with a deletion in exon 19 (del E746–A750) are used, which is well known to be sensitive to EGFR-TKIs [14]. Phosphorylation of Thr-669 and Tyr-1068 was constitutively detected, which was completely suppressed by gefitinib, an EGFR TKI (Fig. 2A). In contrast, pS1046/1047 was not detected due to less activation of p38 (data not shown). To further characterize the mechanism of this signaling network, we used specific inhibitors against ERK and Akt pathways. U0126, but not LY294002, inhibited phosphorylation of Thr-669 without affecting pY1068, indicating that activated EGFR induces constitutive phosphorylation of Thr-669 via ERK activation (Fig. 2B). This was additionally confirmed using HGF, a growth factor causing resistance to EGFR-TKIs, which restored the ERK-dependent Thr-669 phosphorylation, without affecting the EGFR tyrosine phosphorylation (Fig. 2C).

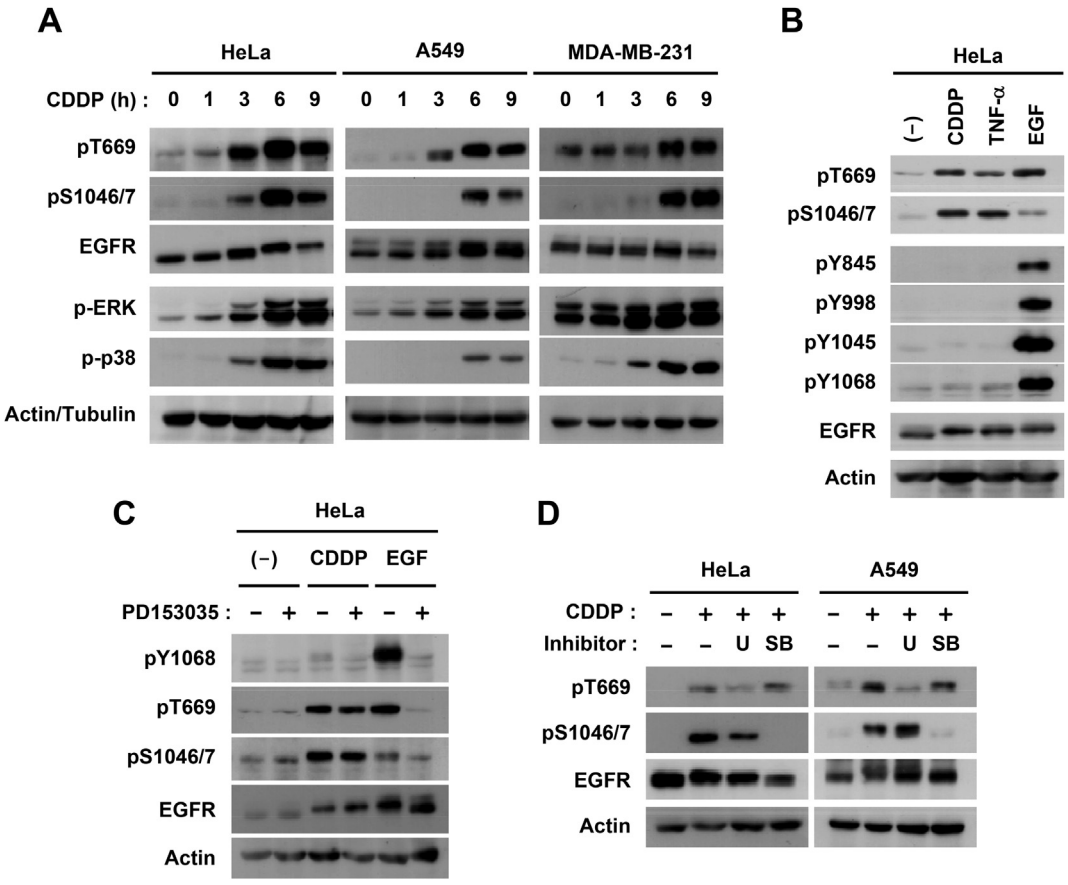

Fig. 1. Effect of CDDP on the activation of EGFR Thr-669 and Ser-1046/1047 in EGFR-wild type cells. (A) HeLa, A549, and MDA-MB-231 cells were treated with 100 μM CDDP for indicated time periods. (B) HeLa cells were stimulated with 100 μM CDDP for 6 h, 20 ng/mL TNF-α for 10 min, or 10 ng/mL EGF for 10 min (C) HeLa cells were pretreated with 1 μM PD153035 for 30 min followed by CDDP for 6 h or EGF for 10 min (D) HeLa and A549 cells were pretreated with 5 μM U0126 or 10 μM SB203580 for 30 min followed by 100 μM CDDP for 6 h. Targeted proteins were detected using immunoblotting. pY, pT and pS mean phospho-tyrosine, phospho-threonine and phospho-serine of EGFR, respectively. Actin or Tubulin was used as loading controls for HeLa/MDA-MB-231 or A549 cells, respectively.

3.3. Cisplatin-induced phosphorylation and endocytosis of EGFR

We next examined the effects of CDDP on phosphorylation of EGFR with activating mutation in PC-9 cells. CDDP induced phosphorylation of Thr-669 and Ser-1046/1047 in a similar time dependent manner to that of wild type EGFR in HeLa cells (Figs. 1A and 3A). In contrast to the increase in serine/threonine phosphorylation, it is interesting that constitutive tyrosine phosphorylation of EGFR decreased gradually (Fig. 3A). To further confirm the role of MAPKs on this downregulation, we evaluated the effect of their inhibitors on EGFR phosphorylation. Inhibition of ERK pathway resulted in selective downregulation of pT669 and restoration of pY1068 from CDDP-induced downregulation (Fig. 3B). This is consistent with our previous finding that Thr-669 is the negative feedback site of tyrosine kinase activity of EGFR homodimer [29]. Moreover, SB203580 completely inhibited CDDP-induced pS1046/1047 with further downregulation of pY1068 (Fig. 3B).

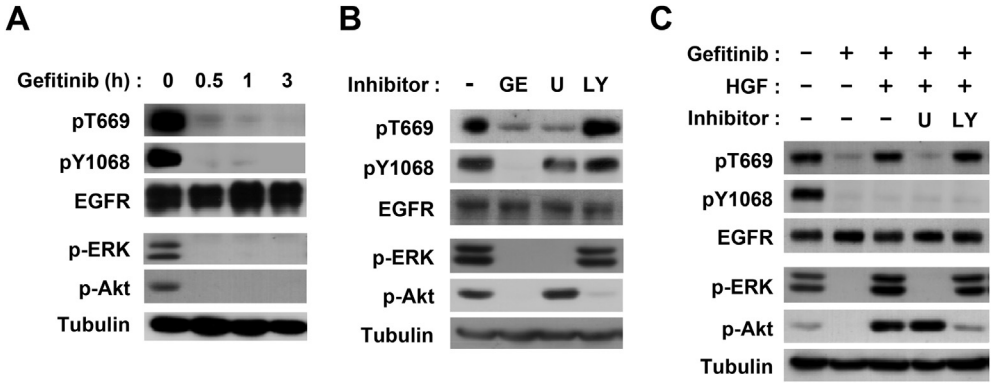

Fig. 2. Exploring EGFR phosphorylation pattern in PC-9 cells in response to different stimuli. (A) PC-9 cells were treated with 0.3 μM gefitinib for the indicated times. (B) PC-9 cells were treated with 0.3 μM gefitinib, 5 μM U0126, or 5 μM LY294002 for 1 h (C) PC-9 cells were treated with gefitinib, U0126, LY294002 and/or 100 ng/mL HGF for 1 h. Targeted proteins were detected using immunoblotting.

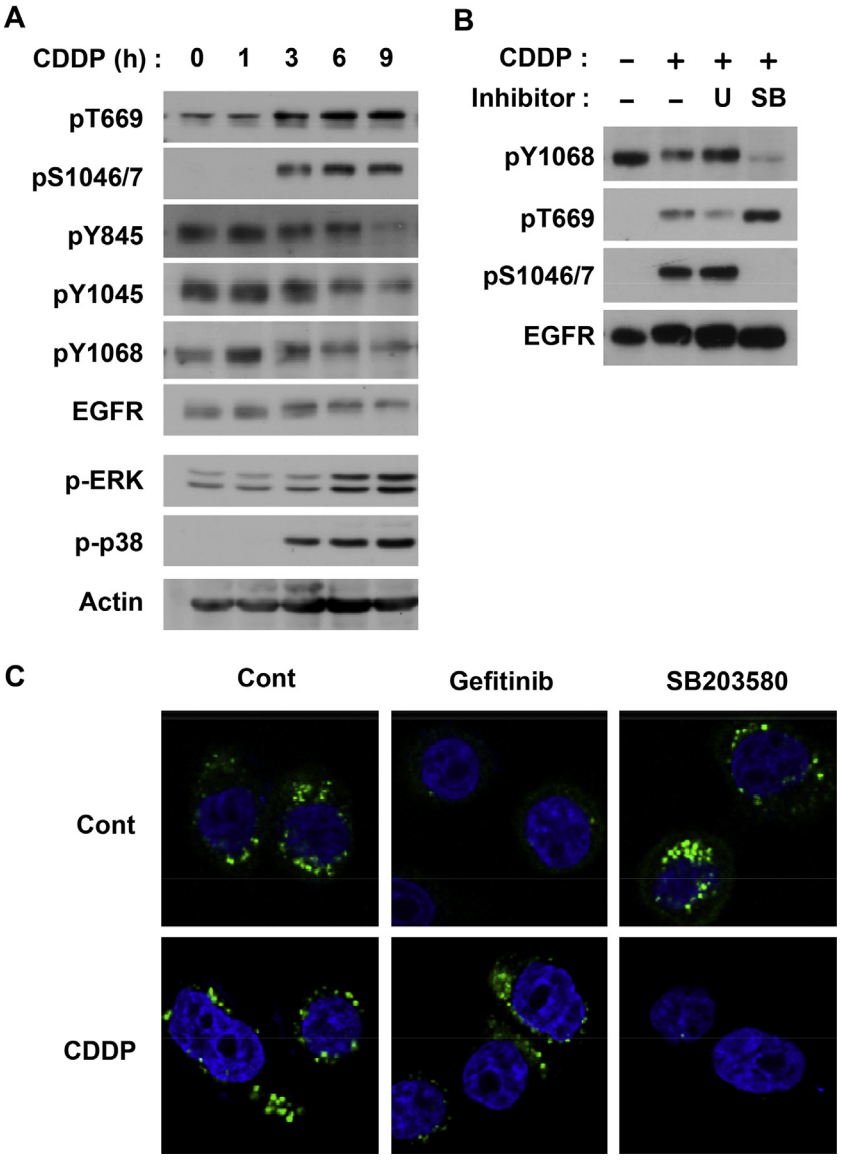

Fig. 3. CDDP-induced EGFR internalization via p38 activation. (A) PC-9 cells were treated with 100 μM CDDP for the indicated times. (B) PC-9 cells pretreated with 5 μM U0126 or 10 μM SB203580 for 30 min followed by 100 μM CDDP for 6 h, and targeted proteins were detected using immunoblotting. (C) PC-9 and RPC-9 cells were pretreated with 1 μM gefitinib or 10 μM SB203580 for 30 min followed by 100 μM cisplatin for 4 h, and EGFR endocytosis was detected using confocal imaging.

It has been demonstrated that p38-dependent phosphorylation of Ser-1046/1047 is suggested to be involved in tyrosine kinase-independent endocytosis of wild type EGFR [15,24,25]. Flow cytometry analysis confirmed TNF-α-induced non-canonical endocytosis of wild type EGFR via p38 activation in A549 cells (Fig. S2). We next investigated CDDP-induced internalization of EGFR with activating mutation in PC-9 cells (Fig. 3C). Immunofluorescence analysis demonstrated that EGFR was constitutively internalized without CDDP treatment in a tyrosine kinase-dependent but not p38-dependent manner. Although 3 h CDDP treatment did not significantly enhanced the level of internalized EGFR protein, it is interesting that internalization mechanism was switched from tyrosine kinase-dependent to p38-dependent non-canonical pathway.

3.4. Role of non-canonical pathway of EGFR in apoptotic cell death in PC-9 cells

Fig. 3 showed that combination of CDDP with SB203580 resulted in strong suppression of EGFR tyrosine phosphorylation as well as complete inhibition of CDDP-induced non-canonical endocytosis of EGFR. We finally evaluated the combination effect on apoptosis. Cleavage of PARP and Caspase-3 was strongly promoted by CDDP with SB203580, but not with U0126, indicating enhanced apoptotic cell death by p38 inhibition (Fig. 4A). This was correlated with a significant reduction in cell viability (Fig. 4B, C). These results suggest the role of p38-mediated phosphorylation of EGFR, in counteracting CDDP-induced apoptosis in cancer cells expressing active mutant EGFR.

4. Discussion

We have shown that various cellular stimuli, including TNF-α and *Helicobacter pylori* infection, trigger the non-canonical EGFR phosphorylation [15,30,31]. In contrast to the tyrosine autophosphorylation-dependent canonical EGFR pathway, the non-canonical pathway is regulated via an intracellular feedback mechanism which is independent of direct ligand/receptor interaction. Apparently, these stimuli employed p38/ERK MAPK

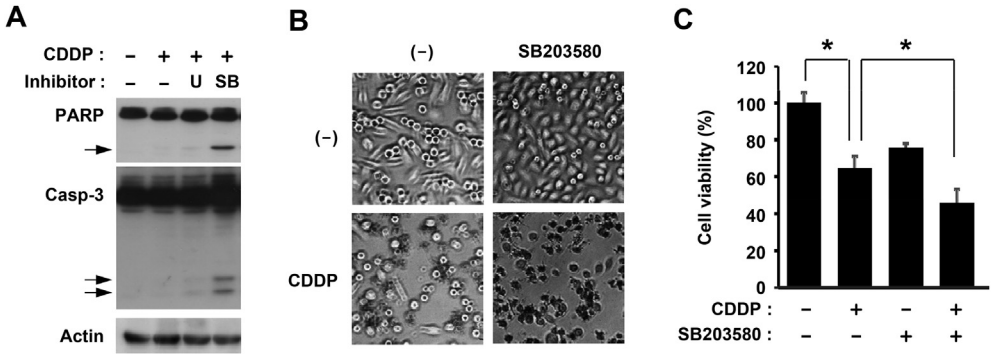

Fig. 4. Inhibition of p38 MAPK enhanced CDDP-induced apoptosis. (A) PC-9 cells were pretreated with 5 μM U0126 or 10 μM SB203580 for 30 min, followed by 100 μM CDDP for 6 h. PARP and Caspase-3 proteins were detected using immunoblotting. Arrows indicate cleaved forms. (B and C) PC-9 cells were pretreated with 10 μM SB203580 for 30 min, followed by 100 μM CDDP for 12 h, and then photos were captured and cell viability was tested in WST-1 assay. *, $P < 0.01$.

pathways to induce feedback phosphorylation of EGFR at serine/threonine residues via a common upstream kinase, transforming growth factor-β-activated kinase 1 (TAK1) (Fig. S1). In this study, we first demonstrated that EGFR harboring active mutation in the kinase domain is also regulated by non-canonical mechanisms even in the presence of gefitinib in human NSCLC cells. These results suggest that serine/threonine phosphorylation plays a role in the regulation of EGFR tyrosine kinase activity, especially in the establishment of acquired resistance to EGFR-TKIs. We, for example, demonstrated that Thr-669 is only phosphorylated in the presence of HGF, a culture condition showing resistance to gefitinib in PC-9 cells.

Although CDDP has been continuously one of the most powerful drugs against several cancers, the current findings uncover a potential explanation for resistance developed in many patients. We demonstrated that CDDP induced phosphorylation of the canonical serine/threonine sites of mutant EGFR. ERK-mediated Thr-669 phosphorylation caused downregulation of constitutively activated EGFR. More importantly, CDDP induced Ser-1046/1047 phosphorylation and subsequent endocytosis of EGFR via p38 pathway even in the presence of gefitinib. We previously reported that p38-mediated endocytosis of EGFR plays an anti-apoptotic role in TNF-α-signaling pathway. In fact, SB203580 promoted CDDP-induced apoptotic responses in PC-9 cells. The combination of agents that target the survival internalization pathway of EGFR with CDDP would be therefore of importance as a newly developed methodology against cancer resistance in NSCLC and other types of cancer.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.044.

Transparency document

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