

Signaling and Regulation

The Combination of Multiple Receptor Tyrosine Kinase Inhibitor and Mammalian Target of Rapamycin Inhibitor Overcomes Erlotinib Resistance in Lung Cancer Cell Lines through c-Met Inhibition

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Abstract

Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI) show antitumor activity in a subset of non-small cell lung cancer (NSCLC) patients. However, the initial tumor response is followed by recurrence. Several studies have suggested the importance of other receptor tyrosine kinases (RTK) and downstream kinases as potential targets in the treatment of NSCLC. We used the multiple-RTK inhibitor AEE788, which inhibits EGFR, vascular endothelial growth factor receptor, and human epidermal growth factor receptor 2, with and without the downstream kinase inhibitor RAD001 (an inhibitor of mammalian target of rapamycin). AEE788 inhibited cell growth more effectively than did erlotinib in three NSCLC cell lines examined (A549, H1650, and H1975). However, in the EGFR-TKI-resistant cell line H1975 harboring T790M resistance mutation, cell growth inhibition by AEE788 was only mild, and the phosphorylation of its leading targets such as EGFR and vascular endothelial growth factor receptor 2 was not inhibited. In H1975, AEE788 induced significantly greater cell growth inhibition when combined with RAD001 than when used alone. This cooperative effect was not seen with the combination of erlotinib and RAD001. We found that c-Met was highly phosphorylated in this cell line, and the phosphorylated c-Met was inhibited effectively by AEE788. Using a phospho-RTK array, the phosphorylation of c-Met and insulin-like growth factor-I receptor was inhibited by AEE788. These results suggest that upstream RTK inhibitor overcomes the acquired resistance to EGFR-TKI when combined with downstream kinase inhibitor. Thus, the combined inhibition of upstream and downstream RTKs is a promising strategy for the treatment of NSCLC. *Mol Cancer Res*; 8(8); 1142–51. ©2010 AACR.

Introduction

In solid tumors, including non-small cell lung cancer (NSCLC), genome-wide screening for gene alterations is thought to be a powerful tool with which to identify promising targets (1–3). Mutations in epidermal growth factor receptor (EGFR) kinase domain were proved to be associated with good clinical responses to the selective EGFR tyrosine kinase inhibitors (EGFR-TKI) gefitinib and erlotinib, especially in lung adenocarcinomas (4, 5). These mutations are

more common in adenocarcinomas, east Asians, women, and never smokers. Response rates exceeding 70% for patients with EGFR mutations have been reported in several prospective clinical trials (6, 7). About 90% of such somatic mutations are clustered in exon 19 (deletion mutation) and exon 21 (point mutation at codon 858) and were found to involve ligand-independent activation and prolonged receptor kinase activity after ligand stimulation (8, 9). However, lung adenocarcinomas with these drug-sensitive EGFR mutations that initially respond to EGFR-TKI eventually develop acquired resistance (10–12). In approximately half of the cases, tumor cells acquired a secondary mutation in exon 20 of the EGFR kinase domain (13, 14).

To overcome the resistance to conventional inhibitors, several studies recently suggested the importance of other receptor tyrosine kinases (RTK), for example, vascular endothelial growth factor receptor (VEGFR) and the downstream RTKs, as potential molecular targets in *in vitro* and *in vivo* studies (15–19). In addition, multiple RTKs are co-activated in many solid tumors, and redundant inputs drive and maintain downstream signaling; thus, targeting single RTKs may limit the efficacy of molecular therapies (20).

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AEE788, a potent multiple-RTK inhibitor, is reported to inhibit several RTKs such as EGFR, ErbB2 [human epidermal growth factor receptor 2 (HER2)], VEGFR2, and other kinases located downstream of these RTKs, and it induces antitumor effects in a subset of solid tumors (21, 22). Mammalian target of rapamycin (mTOR) and its downstream effectors have been also thought to be novel targets for cancer therapeutics (23). mTOR is a conserved serine/threonine protein kinase located in the phosphatidylinositol-3-kinase (PI3K)/Akt pathway, which plays important roles in tumor growth, proliferation, and resistance to apoptosis (24, 25). RAD001 (everolimus), a rapamycin derivative, is a potent inhibitor of mTOR. RAD001 is currently undergoing clinical trials in several solid tumors including NSCLC (26, 27) and was recently approved for the treatment of renal cell carcinoma in the United States.

Many agents have emerged as novel small molecules to inhibit multiple RTKs and their downstream kinases, and the combination of these targeted agents has recently been studied as a potential new strategy for lung cancer therapy. Therefore, we hypothesized that the inhibition of multiple kinases would work more effectively in NSCLC cell lines that have resistance to erlotinib through the inhibition of its various targets such as VEGFR2, HER2, and their downstream kinases, in addition to EGFR. To elucidate the effect and underlying mechanisms, we used AEE788 with and without RAD001 in three different NSCLC cell lines (A549, H1650, and H1975) that have different EGFR sensitivity and EGFR resistance mutation status [i.e., A549 with wild-type EGFR, H1650 with EGFR sensitivity mutation (exon 19 del), and H1975 with EGFR sensitivity (exon 21, L858R) and EGFR resistance (exon 20, T790M) mutations].

Materials and Methods

Cell lines

Three human NSCLC cell lines were used: A549 (EGFR wild-type), NCI-H1650 [H1650; EGFR exon 19 mutation (delE746-A750)], and NCI-H1975 [H1975; EGFR exon 21 mutation (L858R) and exon 20 mutation (T790M); ref. 28]. These cell lines were purchased from the American Type Culture Collection and grown in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum.

Reagents

The cell lines were treated with the following inhibitors as a single agent or in combinations at various concentrations: AEE788 (Novartis Pharma AG), an inhibitor of multiple RTKs such as EGFR, HER2, and VEGFR2; erlotinib (Genentech), an EGFR-TKI; RAD001 (everolimus, Novartis Pharma), an mTOR inhibitor; SU11274 (Calbiochem), a c-Met inhibitor; and tyrphostin AG538 (AG538; Sigma-Aldrich), an insulin-like growth factor-I (IGF-I) receptor (IGF-IR) inhibitor.

Phospho-EGFR (Tyr1068) antibody, total EGFR antibody, phospho-HER2/ErbB2 (Tyr877, Tyr1221/1222) antibody, total HER2/ErbB2 antibody, phospho-c-Met (Tyr1234/1235) antibody, total c-Met antibody, phospho-IGF-IR β (Tyr1131)/phospho-insulin receptor (InsR)- β (Tyr1146) antibody, total IGF-IR β antibody, phospho-p44/42 mitogen-activated protein kinase (MAPK; Tyr202/Tyr204) antibody, total p44/42 MAPK antibody, phospho-Akt (Ser473) antibody, total Akt antibody, phospho-S6K (Ser240/244) antibody, total S6K antibody, β -actin antibody, and antirabbit and antimouse secondary antibodies were purchased from Cell Signaling Technologies.

Cell proliferation assays

For the counting of cell numbers using a Coulter counter (Beckman Coulter), cells were seeded at a density of 1×10^5 per well in 24-well culture plates and allowed to attach for 24 hours. The cells were then treated with AEE788, erlotinib, SU11274, AG538, or RAD001 as a single agent or in combinations at variable concentrations in the presence of 200 ng/mL epidermal growth factor (EGF) and/or 100 ng/mL IGF-I (Sigma-Aldrich), 50 ng/mL hepatocyte growth factor (HGF; R&D Systems), and 100 ng/mL VEGF (Cell Signaling Technologies). Control cells were treated with the same concentration of the vehicle DMSO. Seventy-two hours later, cell number was calculated using a Coulter counter. For the MTS assay (Promega), 5×10^2 cells were seeded per well in 96-well plates and allowed to attach for 24 hours. Seventy-two hours after treatment with the drugs in the same condition as described earlier, cell growth was analyzed according to the manufacturer's protocol.

Western blotting analysis

Cell lines were cultured for 48 hours, serum-starved overnight, and then stimulated with inhibitors at variable concentrations. RTK inhibitors AEE788, erlotinib, SU11274, and AG538 were used at ~ 10 μ mol/L, whereas RAD001 was used at ~ 1 μ mol/L. Drug doses were based on the IC₅₀ data in the cell proliferation assay and available data from other studies. Cell lines were stimulated by these inhibitors for 6 hours and then supplemented with 200 ng/mL EGF, 100 ng/mL VEGF, 50 ng/mL HGF, and/or 100 ng/mL IGF-I for 10 minutes. As a control, cells were treated with the same concentration of the vehicle DMSO. Cells were washed with cold PBS and solubilized in lysis buffer. The suspension was frozen at -80°C . Protein concentrations were calculated and equal amounts of protein were denatured and reduced with sample buffer. After boiling, aliquots of the samples were subjected to electrophoresis. The fractionated proteins were transferred onto Immun-Blot polyvinylidene fluoride membranes (Bio-Rad) at 80 V for 3.5 hours in transfer buffer. Next, the membrane was subjected to the diluted primary antibodies followed by incubation with secondary antibodies. For the detection of proteins, the membrane was incubated with agitation in LumiGLO reagent and peroxide (Cell Signaling Technologies) and exposed to X-ray film.

Phospho-RTK array

A human phospho-RTK array (R&D Systems) was used to detect simultaneously the tyrosine-phosphorylated RTKs. This array contains 42 RTK (such as ErbB family, VEGFR family, and c-Met) capture antibodies spotted in duplicate. Cell lysates (50 μ g) were incubated with the membrane overnight in the buffer provided according to the manufacturer's protocol. Target proteins were captured with their respective antibodies. After washing, the proteins were incubated with a phosphotyrosine antibody conjugated to horseradish peroxidase to allow the detection of captured RTKs that are phosphorylated. Array data on X-ray film images were analyzed using image analysis software (Scion Image, Scion Corporation).

Results

Treatment with AEE788 or RAD001 alone inhibits NSCLC cell proliferation

We performed cell proliferation assay in the three NSCLC cell lines treated with erlotinib, AEE788, or RAD001. Whereas erlotinib inhibited cell proliferation only in H1650 with EGFR deletion (EGFR sensitivity) mutation, AEE788 induced moderate cell growth inhibition in H1650 and A549 with wild-type EGFR and mild but significant inhibition in H1975 with EGFR resistance mutation (Fig. 1). RAD001 also induced significant mild growth inhibition in all the cell lines.

AEE788 does not inhibit its principal target RTKs in erlotinib-resistant cell line

AEE788 inactivated EGFR effectively in A549 cells, similar to erlotinib (Fig. 2A). In H1650 cells, AEE788 induced definite blocking of its targets including HER2 and EGFR as we expected. With respect to H1975, which harbored L858R/T790M mutation, however, AEE788 did not suppress any of these targets. Although VEGFR2

was another main target of AEE788, VEGFR2 was not detected in H1975 by Western blotting analysis. On the other hand, RAD001 induced potent blockage of phosphorylation of p70S6 kinase protein (S6K), which was located downstream of mTOR, in all the cell lines studied (Fig. 2B).

AEE788 cooperates with RAD001 in inhibiting the cell growth of H1975

We attempted combining these two agents and evaluated their effect on cell growth as well as on the phosphorylation status of target RTKs and their downstream kinases. The combined treatment showed no additional effect on growth inhibition compared with AEE788 alone in the H1650 cell line (Fig. 3). Phosphorylation of EGFR, as well as its downstream kinases such as MAPK and Akt, was inhibited sufficiently by AEE788; thus, we assumed that there was no additional effect due to the repression of phosphorylation of S6K with RAD001 (Fig. 4). No additional reduction in cell growth caused by the combination was observed in A549.

Of note, the combination of RAD001 and AEE788 caused a more striking reduction in cell numbers than did either drug alone in the resistant cell line H1975 (Fig. 3).

The IC_{50} was calculated to be 17 μ mol/L for AEE788 and 8 μ mol/L for RAD001 by cell proliferation assay (data not shown). We found synergistic inhibition of cell growth with the combination of these drugs. Whereas 0.1 μ mol/L AEE788 and 1 μ mol/L RAD001 inhibited cell growth by 10.5% and 20.3%, respectively, when used alone, their combination resulted in a 54.9% inhibitory effect. However, phosphorylation of EGFR and its major downstream kinases, such as MAPK and Akt, was not inhibited by the combination of AEE788 and RAD001, with the exception of S6K (Fig. 4). In the study on the main targets of phosphorylation, almost the same results were observed between treatment with AEE788 or RAD001 alone and

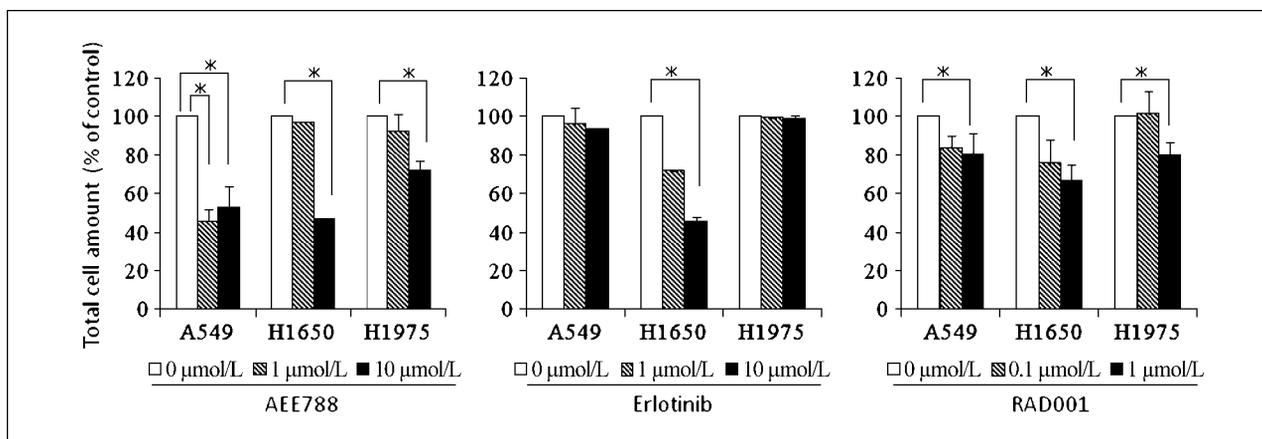
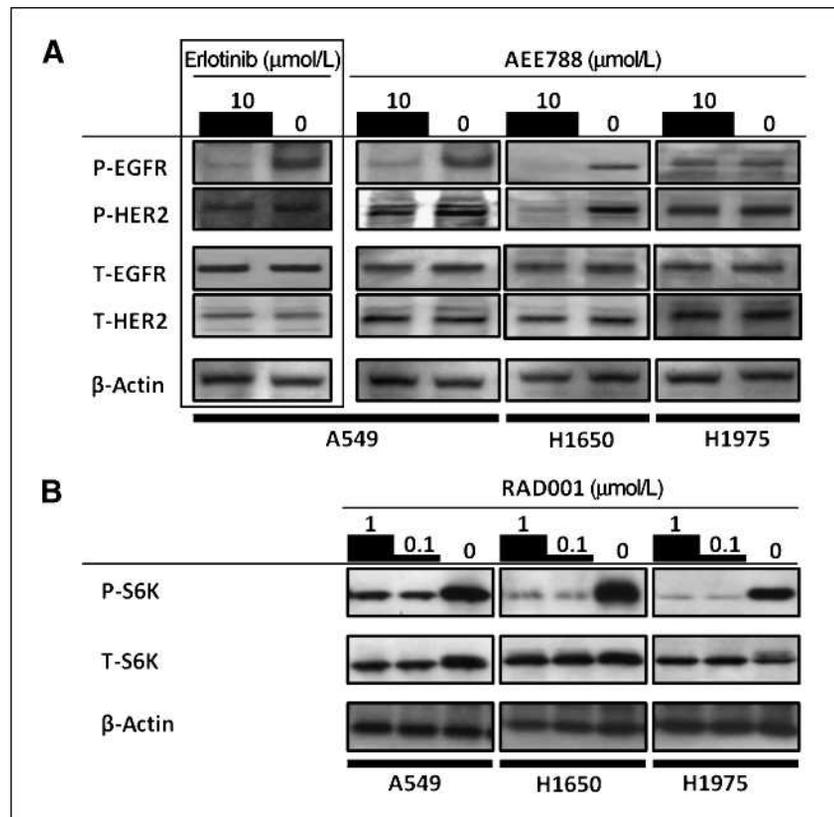


FIGURE 1. Cell proliferation assay of AEE788, erlotinib, or RAD001 treatment alone in the three NSCLC cell lines (left, A549; middle, H1650; right, H1975, in each graph). The cells were incubated in 10% serum-containing medium for 72 h with the drugs at the indicated concentrations. The total cell amount was expressed as the percent of that of control cultures incubated without drugs. Columns, mean of three independent experiments; bars, SD. *, $P < 0.05$, AEE788, erlotinib, or RAD001 versus control (Student's t test).

FIGURE 2. Phosphorylation status of EGFR, HER2, and S6K with or without AEE788 or RAD001 in the three cell lines (left, A549; middle, H1650; right, H1975). Each cell line was stimulated with a single agent, AEE788 (A) or RAD001 (B), at the concentration indicated. A549 was also stimulated with erlotinib as a reference to AEE788 at 10 $\mu\text{mol/L}$. After supplementation with 200 ng/mL EGF for 10 min, the lysates were subjected to Western blot analysis with antibodies to phosphorylated (P-) or total forms (T-) of EGFR, HER2, and S6K.



treatment with their combination in H1975. To confirm such a synergistic effect between AEE788 and RAD001 in the erlotinib-resistant cell line, we compared the differences in the cell growth inhibition induced by the combination of AEE788 and RAD001 with that by erlotinib and RAD001 (Fig. 3). RAD001 plus AEE788 showed more pronounced growth inhibition compared with RAD001 plus erlotinib. Therefore, we assumed that such a synergistic effect achieved by the combination of AEE788 and RAD001 in H1975 was induced by inhibiting other target(s) of AEE788, together with the blockage of the PI3K pathway by RAD001.

As none of the main three RTKs was inhibited by AEE788 in H1975, we examined other potential RTKs and kinases such as Src and signal transducers and activators of transcription-3 (STAT3) to explore the key target of AEE788; however, we did not observe any significant change in the phosphorylation of Src or STAT after the addition of AEE788 (data not shown).

Phospho-RTK array detects potential target RTKs that are inhibited by AEE788

We used a human phospho-RTK array containing 42 RTKs to investigate the relative levels of phosphorylation of different RTKs. We detected that not only EGFR and HER2 but also HGF receptor (c-Met), InsR, and IGF-IR were activated in H1975 (Fig. 5A). Among these RTKs,

we also found that not only c-Met but also InsR and IGF-IR were inhibited by AEE788. Data from the average signals (pixel densities) on each array suggested significant blocking effects for phosphorylated c-Met, InsR, and IGF-IR with AEE788 (Fig. 5B). Insulin receptors are thought to share significant structural and functional similarity with IGF receptors. This array helped to elucidate the receptors effectively inhibited by the AEE788 and RAD001 combination.

c-Met is highly phosphorylated in H1975 and is inhibited effectively by AEE788

We confirmed that c-Met was highly phosphorylated in H1975, and the phosphorylation of c-Met was inhibited effectively by AEE788 even with stimulation by its ligand, HGF (Fig. 6A). Such an inhibitory effect of AEE788 on c-Met phosphorylation was also seen in A549 and H1650. In contrast to the consistency of Western blotting data with RTK array on c-Met, the phosphorylated form of IGF-IR/InsR was not altered in Western blotting (Fig. 6A).

Specific inhibitor of c-Met also shows inhibitory effect on H1975, similar to AEE788

To explore how much influence the specific inhibition of c-Met or IGF-IR had on the activity of the resistant cell line H1975, we introduced SU11274, a specific inhibitor of c-Met, and AG538, an inhibitor of IGF-IR. SU11274

and AG538 effectively inactivated c-Met and IGF-IR, respectively (Fig. 6B). SU11274 showed significantly greater growth inhibition in cell proliferation assay when combined with RAD001 at higher concentration than did AEE788 with RAD001 (Fig. 6C). AG538 had no effect on cell growth even in combination with RAD001 (data not shown) in spite of the sufficient inhibition of phosphorylated IGF-IR.

Discussion

In the present study, the multiple-RTK inhibitor AEE788 was found to induce mild to moderate growth inhibition in the three NSCLC cell lines with different EGFR mutation status. We also showed that AEE788 together with RAD001 had a synergistic growth inhibitory effect in the erlotinib-resistant NSCLC cell line H1975,

partly due to the simultaneous inhibitory effects on c-Met and mTOR.

Multiple-RTK inhibitors are attractive novel agents in the treatment of solid tumors, and a number of these inhibitors are currently undergoing evaluation in several clinical trials (15). The multiple-RTK inhibitor AEE788 has shown antitumor effects in glioblastoma *in vitro* and *in vivo* (22). Therefore, we believe that this agent may be effective in the treatment of NSCLC that has acquired resistance to erlotinib through the inhibition of its various targets. AEE788 monotherapy inhibited cell growth in the three NSCLC cell lines, including the erlotinib-resistant cell line H1975. However, in this cell line, AEE788 exhibited significant but only mild cell growth inhibition. Furthermore, AEE788 failed to inhibit the phosphorylation of its main target RTKs, such as EGFR and HER2. AEE788 was originally reported to show antiproliferative activity

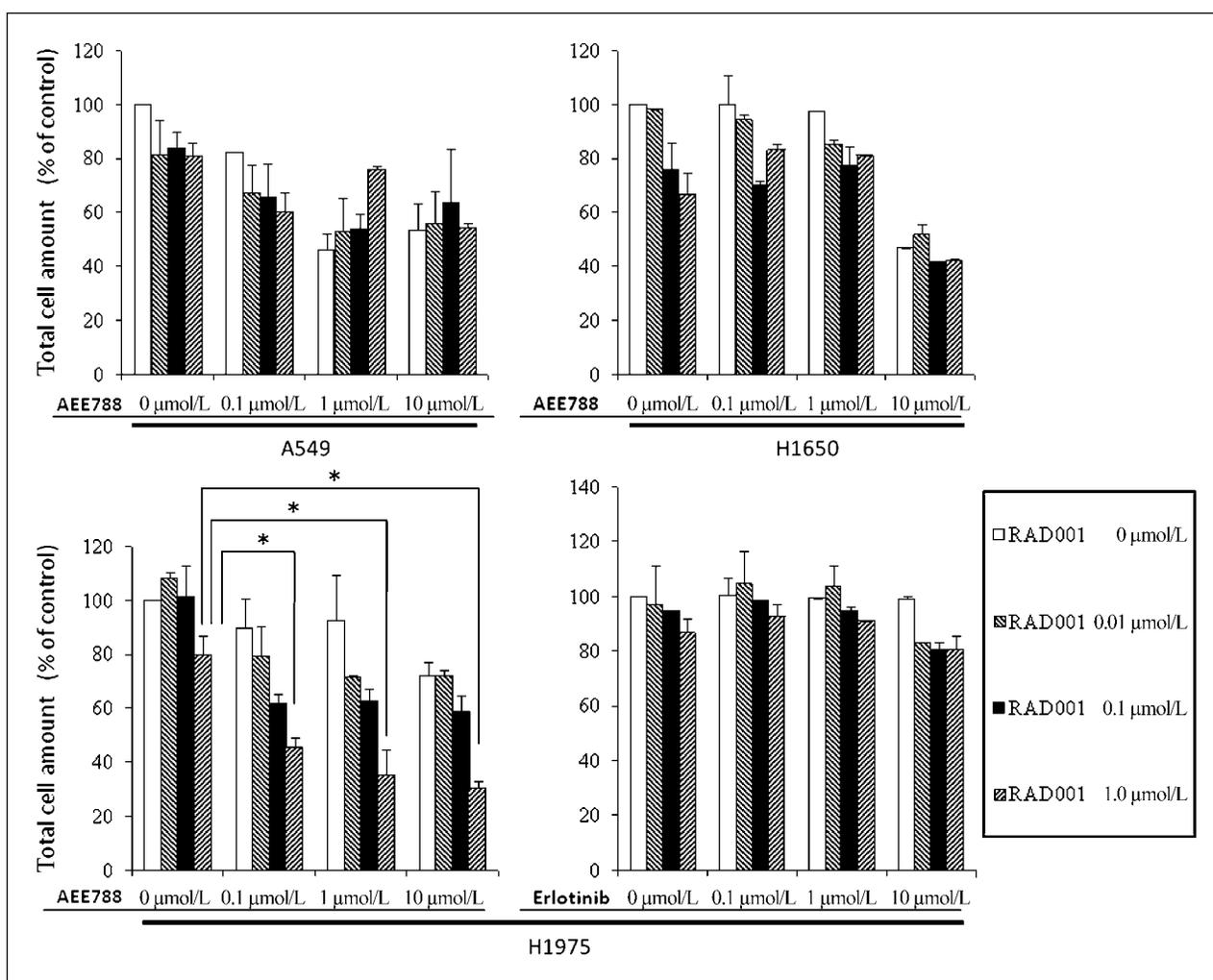


FIGURE 3. Cell proliferation assay of the combination of AEE788 and RAD001 or erlotinib and RAD001 in the three cell lines (top left, A549; top right, H1650; bottom left, H1975 with AEE788 and RAD001; bottom right, H1975 with erlotinib and RAD001). Each cell line was grown in 24-well plates in 10% fetal bovine serum-containing medium for 72 h. The total cell amount is expressed as the percent of that of control cultures without drugs. Columns, mean of three independent experiments; bars, SD. *, $P < 0.05$, AEE788 plus RAD001 (1 μmol/L) versus RAD001 (1 μmol/L) alone (Student's *t* test).

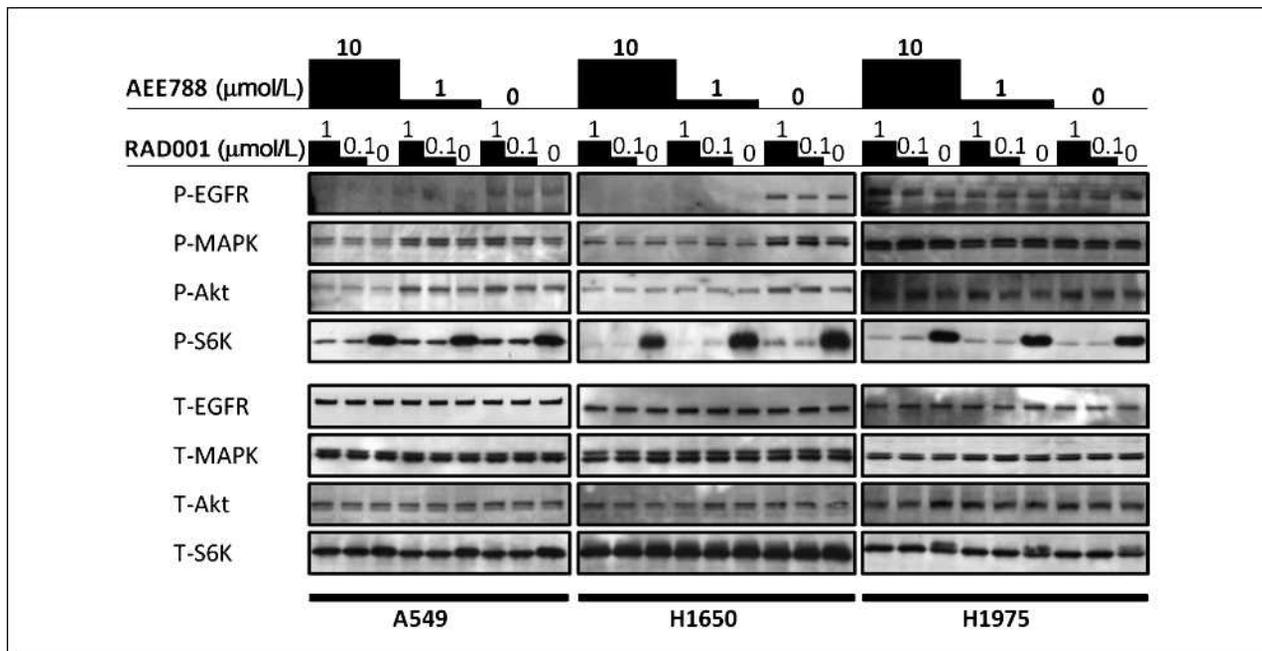


FIGURE 4. Phosphorylation status of EGFR and the downstream kinases MAPK, Akt, and S6K (left, A549; middle, H1650; right, H1975). Each cell line was incubated for 6 h with the combination of AEE788 and RAD001 at the indicated concentrations in medium containing 10% serum. After supplementation with 200 ng/mL EGF for 10 min, the lysates were subjected to Western blot analysis with antibodies to phosphorylated or total forms of EGFR, MAPK, Akt, and S6K.

through the inhibition of EGFR, HER2, and VEGFR (mainly VEGFR2) at the nanomolar range (21). However, the phosphorylated forms of EGFR and HER2 were not inhibited by AEE788. Although it has been suggested that about a fourth or a fifth of lung cancer cell lines retain activated VEGFR2 (28), phosphorylated and total forms of VEGFR2 were not detected in H1975 by Western blotting analysis in spite of the stimulation with VEGF. Thus, the antitumor effect caused by AEE788 in the erlotinib-resistant cell line may result from the inhibition of other RTKs.

Cell proliferation of the H1650 cell line with EGFR sensitivity mutation, which also has phosphatase and tensin homologue (*PTEN*) loss (29), was reduced adequately by AEE788 alone because of the effective blocking of EGFR and HER2. In the EGFR wild-type cell line A549, cell growth reduction was also mildly achieved by AEE788 with the inhibition of MAPK and Akt, most likely through the inactivation of EGFR.

On the other hand, RAD001, an mTOR inhibitor, induced mild growth inhibition when used alone in all three cell lines with effective blocking of phosphorylation of S6K, which is located downstream of mTOR. Inhibiting the PI3K/Akt/mTOR pathway is reported to be very important for the induction of apoptosis and the regulation of angiogenesis in cancer cells (30-33). However, it is also reported that inhibition of mTOR alone resulted in upstream Akt activation, and this feedback loop may attenuate the therapeutic effects of mTOR inhibitors (25, 34). Although RAD001 is under clinical evaluation in several

solid tumors, such downstream kinase inhibitors may have limited effect due to feedback mechanisms.

We hypothesized that inhibiting multiple targets simultaneously, including upstream activator receptors and downstream mediator kinases, would be necessary to overcome NSCLC. As we have shown above, the combination of RAD001 and AEE788 induced marked synergistic growth inhibition in H1975, whereas the combination resulted in no more additional effect of RAD001 compared with AEE788 alone in A549 and H1650. This interesting antitumor effect may work through the inhibition of other unknown targets of AEE788, together with blockage of the PI3K pathway by RAD001. At first, we hypothesized that c-Src would be a main target of AEE788 because Src-family kinases function as gatekeepers for many signaling pathways and have been an important prosurvival mediator of EGFR by activating STAT3, which is located downstream of RTKs (35, 36). Furthermore, activated c-Src was also reported to be inhibited by AEE788 at the nanomolar range (21). However, neither phosphorylated c-Src nor phosphorylated STAT3 was inhibited by AEE788 in H1975.

Next, as another potential target of AEE788, we examined c-Met, a unique RTK that is activated via its natural ligand, HGF (37). Activated c-Met is responsible for triggering a number of intracellular signaling cascades and plays important roles in cell proliferation, survival, migration, and angiogenesis (38). This RTK is also found to be overexpressed in up to 67% of lung adenocarcinomas (39, 40). In NSCLC cell lines, including H1975, Tang et al. previously reported signaling of cross-activation functions

between the *c*-Met and EGFR pathways. They demonstrated EGF-induced *c*-Met phosphorylation and the cooperative effect of *c*-Met inhibitor and EGFR-TKI in H1975 cells (39). Furthermore, *MET* amplification and/or phosphorylated *c*-Met has been reported to account for resistance in EGFR-dependent cancers after treatment with EGFR-TKI (41-43). The EGFR-TKI-resistant cell line may be dependent on the *c*-Met pathway for its proliferation and/or survival and be sensitive to the targeted inhibition of *c*-Met, which is thought to be the “addicting oncogene” (29, 44). Although *c*-Met is not a leading target of AEE788 and we could not elucidate the entire mechanism for the combined inhibitory effect of AEE788 and RAD001, inhibiting these cross-activated upstream RTKs by AEE788 together with the blocking of mTOR by RAD001 resulted in the synergistic growth inhibition of H1975. An area of growing interest is this cross-activation

between the EGFR pathway and the other signal pathways, which might explain an escape mechanism in the presence of EGFR-TKI (39, 45, 46). In particular, several studies suggested that the *c*-Met pathway could replace the EGFR pathway by amplification and overexpression in EGFR-TKI-resistant cells.

We used an RTK array to detect the relative levels of phosphorylation of different RTKs that have been effectively inhibited by AEE788 in H1975. This array is a rapid and sensitive tool that is used to simultaneously identify the phosphorylation status of 42 different RTKs. Here, we confirmed that the phosphorylated *c*-Met was effectively inhibited when AEE788 was added. In addition, activated *c*-Met was also inhibited by SU11274, and this inactivation resulted in an antiproliferative effect in combination with RAD001, although it required relatively higher concentration of RAD001 compared with the combination with AEE788.

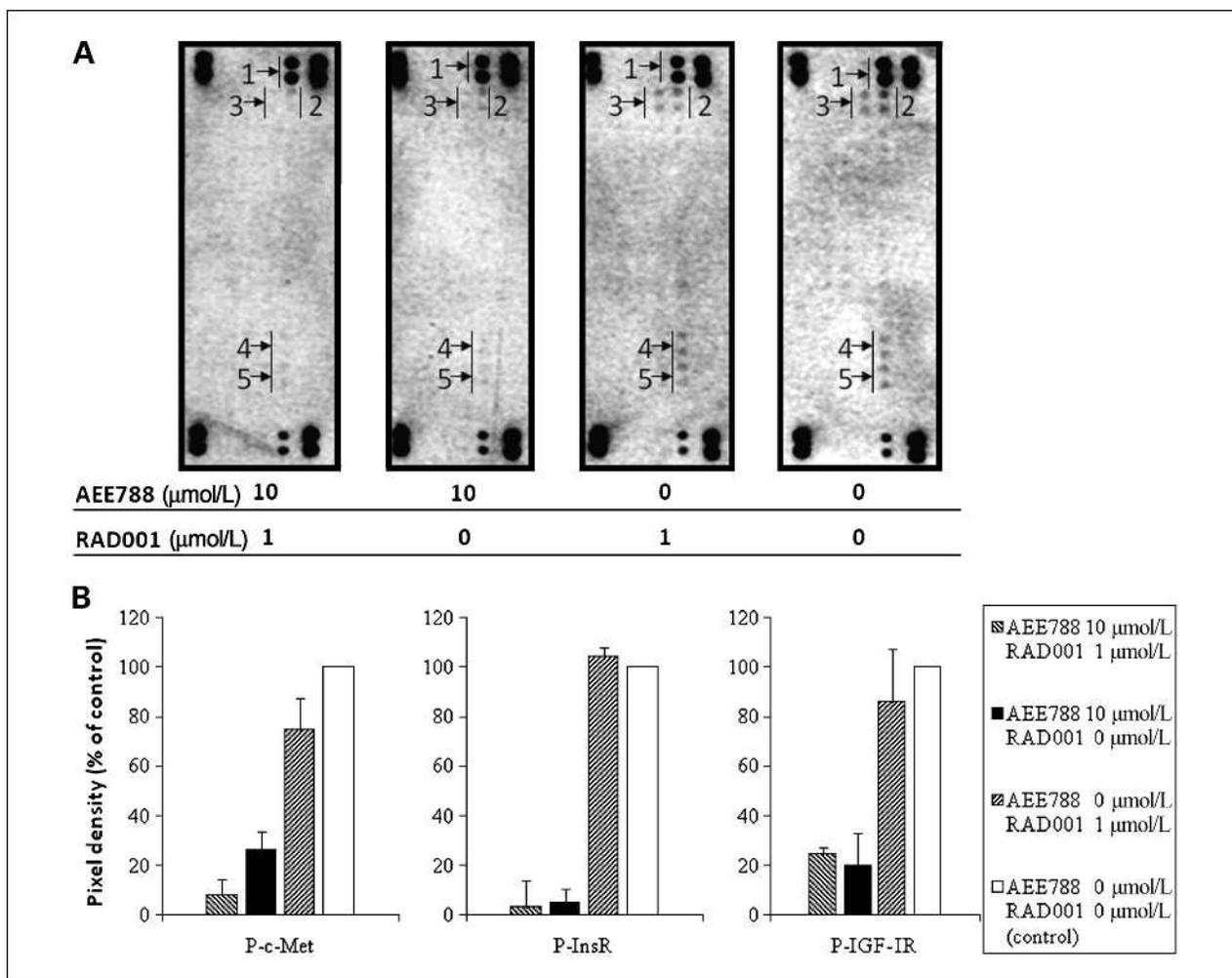


FIGURE 5. Simultaneous detection of the phosphorylation status of 42 RTKs in H1975 cell line using human phospho-RTK array. A, the array detected the tyrosine-phosphorylated RTKs simultaneously in duplicate (1, phosphorylated EGFR; 2, phosphorylated HER2; 3, phosphorylated *c*-Met; 4, phosphorylated InsR; 5, phosphorylated IGF-IR). Duplicate dots in each corner are positive controls. B, the average array signals (pixel densities) from scanned film images were analyzed and indicated as graphs. Pixel densities are expressed as percent of the density of control after subtraction of an averaged background signal from each RTK spot. P-*c*-Met, P-InsR, and P-IGF-IR indicate phosphorylated forms of *c*-Met, InsR, and IGF-IR, respectively.

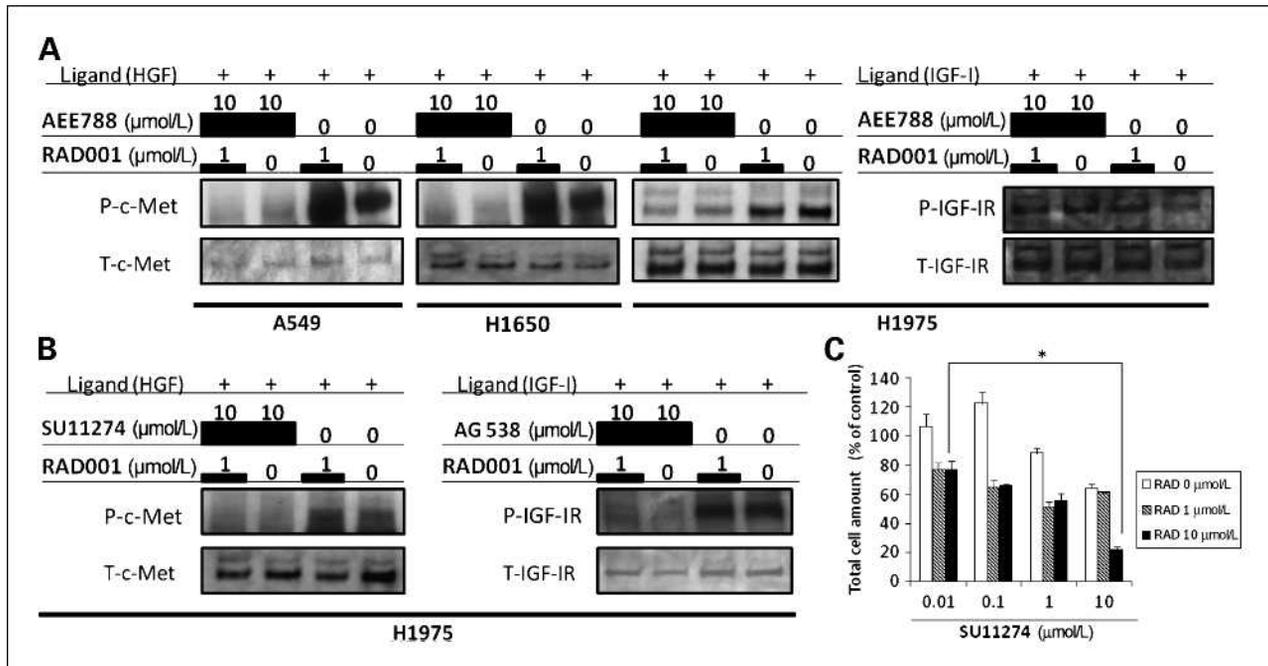


FIGURE 6. Phosphorylation analysis of c-Met and IGF-IR with the combination of AEE788 + RAD001 in the three cell lines (A) and with SU11274 + RAD001 or AG538 + RAD001 in H1975 (B) in a ligand-stimulated manner. The cell lines were incubated for 6 h with these agents at the concentrations indicated. After supplementation for 10 min with 50 ng/mL HGF or 100 ng/mL IGF-I as a ligand, the lysates were subjected to Western blot analysis with antibodies to phosphorylated or total forms of those RTKs. C, cell proliferation assay of the combination of SU11274 and RAD001 in H1975. Cells were grown with these drugs in medium containing 50 ng/mL HGF. The total cell amount is expressed as the percent of that of control culture without drugs. Columns, mean of three independent experiments; bars, SD. *, $P < 0.05$, SU11274 (10 $\mu\text{mol/L}$) plus RAD001 (10 $\mu\text{mol/L}$) versus SU11274 (0.01 $\mu\text{mol/L}$) plus RAD001 (10 $\mu\text{mol/L}$; Student's t test).

These data indicate that AEE788 is one of the effective direct inhibitors of c-Met.

On the other hand, IGF-IR and InsR are also reported to be overexpressed and/or phosphorylated in many types of solid tumors. As one of the promising molecular agents, IGF-IR inhibitors are currently in clinical trials for several solid tumors (47). As seen in the c-Met pathway, some studies have shown that IGF-IR also interacts with EGFR to maintain cell survival (48, 49). IGF-IR and InsR were also effectively inhibited by AEE788 in an RTK array; however, we could not confirm by Western blot analysis if the inhibition of phosphorylated IGF-IR was IGF-I dependent. Together with the negative result of growth inhibition by the combination of AG538 and RAD001, it seemed that IGF-IR was less likely to be involved in the AEE788 inhibition of H1975 cell growth. Nevertheless, we could not fully exclude the possibility of cross-talk between EGFR or mTOR and IGF-IR (InsR) as well as c-Met, considering that dual inhibition of c-Met and mTOR required higher concentration of RAD001 relative to the combination of AEE788 and RAD001 to obtain the same growth inhibitory effect. Extremely diverse and complicated cross-talk mechanisms are supposed to exist between these members of RTK families. Although this is beyond the scope of current experiments, combined targeted therapy for these potential targets, such as c-Met, IGF-IR, InsR, and/or mTOR, would be worth pursuing in the treatment of NSCLC in the future. There may be a more convincing

mechanism by which the combination of upstream RTK inhibitor and downstream mTOR inhibitor is effective in the treatment of resistant tumors. In our research, we have only studied some of the RTKs that have potential in the treatment of NSCLC. Further investigations will be required by studying many other RTKs, the cross-activations between them, and the connection of upstream receptors and their downstream protein kinases to overcome EGFR-TKI resistance.

To summarize, cooperation between AEE788 and RAD001 resulted in synergistic cell growth inhibition in erlotinib-resistant NSCLC cell lines. One of the mechanisms of this anticancer effect may be achieved by the inhibition of c-Met and other potential kinases by AEE788. Here, we suggest that the combination of an upstream RTK inhibitor and its downstream kinase inhibitor may be a promising strategy in lung cancer therapy to overcome resistance to EGFR-TKI.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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