The combination of multiple receptor tyrosine kinase inhibitor and mTOR inhibitor overcomes erlotinib-resistant lung cancer cell line through c-Met inhibition

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Running Title

Inhibition of multiple RTKs and mTOR in resistant NSCLC

Key words (5 words)

c-Met, EGFR, AEE788, RAD001, NSCLC
Abstract

The epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) show anti-tumor 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 (RTKs) 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 (VEGFR), and HER2 with and without the downstream kinase inhibitor RAD001 (an inhibitor of mammalian target of rapamycin (mTOR)). AEE788 inhibited the cell growth more effectively than erlotinib in the three NSCLC cell lines examined (A549, H1650, H1975). However, in the EGFR-TKI resistant cell line H1975 harboring T790M resistant mutation, cell growth inhibition by AEE788 was only mild, and the phosphorylation of its leading targets such as EGFR and VEGFR2 was not inhibited. In H1975, AEE788 induced significant cell growth inhibition when combined with RAD001 compared to when used alone. This cooperative effect was not seen in the combination of erlotinib with 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-1 receptor was inhibited by AEE788. These results suggest that inhibiting upstream RTKs overcomes the acquired resistance to EGFR-TKI when combined with an inhibitor of downstream kinase. Thus, the combined inhibition of upstream and downstream RTKs is a promising strategy for the treatment of NSCLC.
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 (RTKs), 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, so targeting single RTKs may limit the efficacy of molecular therapies (20). AEE788, a potent multiple RTK inhibitor, is reported to inhibit several RTKs such as EGFR, ErbB2 (HER2), VEGFR2, and other kinases located downstream of these RTKs, and it induces anti-tumor 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 in 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
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 both with and without RAD001 in three different NSCLC cell lines (A549, H1650, H1975), which have different EGFR-sensitive and resistant mutation status, i.e. A549 with wild type EGFR, H1650 with EGFR sensitive mutation (exon 19 del), and H1975 with EGFR sensitive (exon 21, L858R) and resistant (exon 20, T790M) mutations.
Materials & Methods

Cell lines

Three human NSCLC cell lines were used: A549: EGFR wild type, NCI-H1650 (H1650): EGFR exon19 mutation (delE746-A750) and NCI-H1975 (H1975): EGFR exon21 mutation (L858R) and exon20 mutation (T790M) (28). These cell lines were purchased from American Type Culture Collection (Manassas, VA), and grown in Dulbecco’s Modified Eagle Medium (Gibco BRL, Gaithersburg, MD) 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, Basel, Switzerland): a multiple RTK inhibitor such as EGFR, HER2 and VEGFR2, erlotinib (Genentech, San Francisco, CA): an EGFR-TKI, RAD001 (everolimus, Novartis Pharma): an mTOR inhibitor, SU11274 (Calbiochem, Cambridge, MA): a hepatocyte growth factor receptor (c-Met) inhibitor, and Tyrphostin AG538 (AG538; Sigma-Aldrich,
Saint Louis, MO: an insulin-like growth factor-1 receptor (IGF-1R) inhibitor.

Phospho-EGF receptor (Tyr1068) antibody, total EGF receptor antibody, phospho-HER2/ErbB2 (Tyr877, Tyr1221/1222) antibody, total HER2/ErbB2 antibody, phospho-c-Met (Tyr1234/1235) antibody, total c-Met antibody, phospho-IGF-1 receptor beta (Tyr 1131)/phospho-Insulin receptor beta (Tyr 1146) antibody, total IGF-1 receptor beta 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, beta-Actin antibody and anti-rabbit and anti-mouse secondary antibodies were purchased from Cell Signaling Technologies (Beverly, MA).

*Cell proliferation assays*

When counting cell numbers with Coulter counter (Beckman Coulter, Fullerton, CA), cells were seeded at a density of 1x10^5/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 as a combination at variable concentrations in...
the presence of 200 ng/ml epidermal growth factor (EGF) and/or 100 ng/ml insulin-like growth factor-1 (IGF-1) (Sigma-Aldrich), 50 ng/ml hepatocyte growth factor (HGF) (R&D Systems, Minneapolis, MN), and 100 ng/ml vascular endothelial growth factor (VEGF) (Cell Signaling Technologies). Control cells were treated with the same concentration of the vehicle dimethyl sulfoxide (DMSO). Seventy-two hours later, the cell number was calculated using a Coulter counter. When using MTS assay (Promega, Madison, WI), 5×10^2 cells were seeded per well in 96-well plates and allowed to attach for 24 hours. 72 hours after treatment of drugs in the same condition above, 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 the following inhibitors at variable concentrations. RTK inhibitors as AEE788, erlotinib, SU11274 and AG538 were used at ~10µM, while RAD001 was used at ~1µM. Drug doses were based on the IC_{50} 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, and/or 50 ng/ml HGF, 100 ng/ml IGF-1 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, Richmond, CA) at 80V 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 µg)
were incubated with the membrane overnight in the buffer provided according to the manufacturer's protocol. Target proteins were captured by their respective antibodies. After washing, incubation with a phosphotyrosine antibody conjugated to horseradish peroxidase allows the detection of captured RTKs which are phosphorylated. Array data on X-ray film images were analyzed using image analysis software (Scion Image, Scion Corporation, Frederick, MD).
Results

Single use of AEE788 or RAD001 inhibited NSCLC cell proliferation

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

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

AEE788 inactivated EGER effectively in A549 cells just like as 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 cell growth of H1975**

We attempted combining these two agents and evaluated the effect on cell growth as well as the phosphorylation status of target RTKs and their downstream kinases.

This combined treatment showed no additional effect on growth inhibition, compared to 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, so 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 the effects of either drug alone in the resistant cell line H1975 (Fig. 3).

The IC$_{50}$ for each agent was calculated to be 17µM for AEE788 and 8µM for RAD001 by cell proliferation assay (data not shown). Then we found synergistic inhibition of cell growth.
growth in the simultaneous presence of these drugs. While 0.1µM AEE788 and 1µM RAD001 inhibited cell growth by 10.5% and 20.3%, respectively when used alone, their combination resulted in 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 regarding the main targets of phosphorylation, almost the same results were observed between the single use and the combined use of AEE788 and RAD001 in H1975. In order 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 of erlotinib and RAD001 (Fig. 3). RAD001 plus AEE788 showed more pronounced growth inhibition than 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 3 RTKs were 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 didn't observed any significant change of phosphorylation of Src or STAT after the addition of AEE788 (data not shown).

*Phospho-RTK array detected potential target RTKs which were 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 c-Met, insulin receptor (InsR) and IGF-1R were activated in H1975 (Fig. 5A). Among these RTKs, we also found, not only c-Met, but also InsR and IGF-1R 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-1R with AEE788 (Fig. 5B). Insulin receptors are thought to share the significant structural and functional similarity with IGF receptors. This array helped to elucidate the effectively inhibited receptors by AEE788 and RAD001 at the same time.

*c-Met is highly phosphorylated in H1975, and 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 in the stimulation with its ligand, HGF (Fig. 6A). Such an inhibitory effect of AEE788 on the c-Met phosphorylation was also seen in A549 and H1650. In contrast to the consistency of Western blotting data with RTK array regarding c-Met, phosphorylated form of IGF-1R/InsR was not altered in Western blotting (Fig. 6A).

Specific inhibitor of c-Met also showed inhibitory effect on H1975 like AEE788

To explore how much influence the specific inhibition of c-Met or IGF-1R had on the activity of the resistant cell line, we introduced SU11274, a specific inhibitor of c-Met, and AG538, an inhibitor of IGF-1R. Both SU11274 and AG 538 effectively inactivated c-Met and IGF-1R, respectively (Fig. 6B). SU11274 brought the significant growth inhibition in cell proliferation assay when combined with RAD001 in higher concentration than AEE788 with RAD001 (Fig. 6C). While, AG538 had no effect on cell growth even in the combination with RAD001 (data not shown) in spite of the sufficient inhibition of phosphorylated IGF-1R.
Discussion

In the present study, the multiple-RTK inhibitor AEE788 was found to induce mild to moderate growth inhibition in three NSCLC cell lines with different EGFR mutation status. We also demonstrated that AEE788 along with RAD001 had a synergistic growth inhibitory effect in the erlotinib-resistant NSCLC cell line H1975, partly due to the simultaneous inhibitory effect on c-Met and mTOR.

Multiple-RTK inhibitors are attractive novel agents for treating solid tumors, so a number of these inhibitors are currently undergoing evaluation in several clinical trials (15). A multiple-RTK inhibitor AEE788 has shown an anti-tumor effect in glioblastoma in vitro and in vivo (22). Therefore, we believe this agent may be effective in the treatment of NSCLC having acquired resistance to erlotinib through inhibition of its various targets. AEE788 monotherapy inhibited the cell growth in the 3 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 demonstrate antiproliferative activity.
through the inhibition of EGFR, HER2, and VEGFR (mainly VEGFR2) at the nM range (21). However, the phosphorylated form of EGFR and HER2 was not inhibited by AEE788. Although it has been suggested that about a fourth or a fifth of lung cancer cell lines retained 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 anti-tumor effect caused by AEE788 in the erlotinib-resistant cell line may result from the inhibition of other RTKs.

Cell proliferation in the H1650 cell line with EGFR sensitive 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 mainly 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 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, while the combination resulted in no more additional effect of RAD001 compared to AEE788 alone in A549 and H1650. This interesting anti-tumor effect may work via the inhibition of other unknown targets of AEE788, together with blockage of the PI3K pathway by RAD001. At first, we hypothesized c-Src would be a main target of AEE788 because Src family kinases (SFK) 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 nM 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 overexpressed in up to 67% of lung adenocarcinomas (39, 40). In NSCLC cell lines, including H1975, Tang et al. previously reported that signaling cross-activation functions between c-Met and EGFR pathways. They demonstrated that 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 have 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 EGFR pathway and other signal pathways, which might explain an escape mechanism in the presence of EGFR-TKI (39, 45, 46). In particular, several studies suggested that, c-Met pathway could replace the EGFR pathway by amplification and overexpression in resistant cells to EGFR-TKI.

We used an RTK array in order to detect the relative levels of phosphorylation of different RTKs which have been effectively inhibited by AEE788 in H1975. This array is a rapid and sensitive tool which 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 brought anti-proliferative effect in the combination with RAD001 although it required relatively higher concentration of RAD001 compared to the combination with AEE788. These data indicate that AEE788 is one of the effective direct inhibitors of c-Met.
On the other hand, IGF-1R and InsR are also reported to be over-expressed and/or phosphorylated in many types of solid tumors. As one of the promising molecular agents, clinical trials of IGF-1R inhibitors are currently ongoing in several solid tumors (47). As seen in c-Met pathway, some studies have demonstrated that IGF-1R and EGFR pathway can also affect each other to maintain cell survival (48, 49). IGF-1R and InsR were also effectively inhibited by this agent in RTK array, however, we could not confirm the distinct inhibition of phosphorylated IGF-1R in the IGF-1-stimulated manner in Western blotting, disappointingly. Together with the negative result of growth inhibition by the combination of AG538 and RAD001, it seemed that IGF-1R was less likely to be involved for AEE788 to inhibit cell growth in H1975 cells. Nevertheless, we could not fully exclude the possibility of cross-talk among EGFR or mTOR and IGF-1R (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 effect on growth inhibition. Extremely diverse and complicated “cross-talk” mechanisms are supposed to exist between these members of RTK families. Even though this is beyond the scope of current experiments, combined targeted therapy for
these potential targets, such as c-Met, IGF-1R, 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, cross-activations between them, and the connection of upstream receptors and their downstream protein kinases in order to overcome the resistance.

To summarize, cooperation between AEE788 and RAD001 resulted in synergistic cell growth inhibition in an erlotinib-resistant NSCLC cell line. One of the mechanisms of this anti-cancer 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 for lung cancer therapy to overcome resistance to EGFR-TKI.
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Figure legends

Figure 1  Cell proliferation assay performed by single use of AEE788, erlotinib, or RAD001 in the 3 NSCLC cell lines (left : A549, middle : H1650, right : H1975 in each graph). The cells were incubated in medium containing 10% serum for 72 hours at the drug concentration indicated. The total cell amount was expressed as the % of that of control cultures incubated without drug. Data are mean values of three independent experiments. *, P <0.05 for the use of AEE788, erlotinib, or RAD001 versus control (Student’s t test). Bars indicate standard deviation.

Figure 2  Phosphorylation status of EGFR, HER2, and S6K with or without AEE788 or RAD001 in the 3 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 10µM. After supplementation with 200 ng/ml EGF for 10 minutes, the lysates were subjected to Western blot analysis with antibodies to phosphorylated (P-) or total forms (T-) of EGFR, HER2 and S6K.
Figure 3  Cell proliferation assay of the combination of AEE788 with RAD001, or erlotinib with RAD001 in the three types of cell lines (upper left: A549, upper right: H1650, lower left: H1975 with AEE788 and RAD001, lower right: H1975 with erlotinib and RAD001). Each cell line was grown in 24-well plates in 10% FBS-containing medium for 72 hours. The total cell amount is expressed as the % of that of control cultures without drugs. Data are mean values of three independent experiments. *, $P < 0.05$ for the combination of AEE788 plus RAD001 (1µM) versus RAD001 (1µM) alone (Student’s $t$ test). Bars indicate standard deviation.

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 hours 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 minutes, the lysates were subjected to Western blot analysis with antibodies to phosphorylated (P-) or total forms (T-) of EGFR, MAPK, Akt and S6K.
Figure 5  Simultaneous detection of phosphorylations of 42 RTKs in H1975 cell line using human phospho-RTK array. (A) This 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-1R). 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 % of the density of control after subtraction of an averaged background signal from each RTK spot. P-c-Met, P-InsR and P-IGF-1R indicate phosphorylated forms of c-Met, InsR and IGF-1R, respectively.

Figure 6  Phosphorylation analysis of c-Met and IGF-1R with the combination of AEE788 + RAD001 in 3 cell lines (A), and SU11274 or AG538 + RAD001 in H1975 (B) in ligand-stimulated manner. The cell lines were incubated for 6 hours with these agents at the concentrations indicated. After supplementation with 50 ng/ml HGF or 100 ng/ml IGF-1 for 10 minutes as a ligand, the lysates were subjected to Western blot analysis.
with antibodies to phosphorylated (P-RTKs) or total forms (T-RTKs) of those RTKs. (C) Cell proliferation assay performed by the combination of SU11274 and RAD001 in H1975. Cells were grown in 50ng/ml HGF ligand containing medium with these drugs. The total cell amount is expressed as the % of that of control culture without drugs. Data are mean values of three independent experiments. *, P < 0.05 for the combination of SU11274 (10µM) plus RAD001 (10µM) versus RAD001 (10µM) alone (Student’s t test). Bars indicate standard deviation.
References


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<td>RAD001 (μM)</td>
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| AEE788 10μM, RAD001 1μM |
| AEE788 10μM, RAD001 0μM |
| AEE788 0μM, RAD001 1μM |
| AEE788 0μM, RAD001 0μM (control) |
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