Synergistic cell growth inhibition by the combination of amrubicin and Akt-suppressing agents in *K-ras* mutation-harboring lung adenocarcinoma cells: Implication of EGFR tyrosine kinase inhibitors

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Received October 7, 2013; Accepted November 26, 2013

DOI: 10.3892/ijo.2014.2249

Abstract. Previously we showed that Akt-suppressing agents, combined with amrubicin, synergistically inhibited the growth of small cell lung cancer cells. The combined effects of chemotherapeutic agents and Akt-suppressing agents, including epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors, were evaluated in A549 lung adenocarcinoma cells harboring *K-ras* mutation and wild-type *EGFR*. Only amrubicin and not other chemotherapeutics (cisplatin, pemetrexed and paclitaxel) synergistically inhibited cell growth when combined with an Akt inhibitor, LY294002. The combination of amrubicin and LY294002 enhanced Annexin V binding to cells. A non-specific tyrosine kinase inhibitor, genistein, suppressed Akt and showed synergistic interaction in combination with amrubicin. Two EGFR tyrosine kinase inhibitors (EGFR-TKIs), gefitinib and erlotinib, suppressed Akt activity at clinically achievable concentrations and demonstrated synergism when combined with amrubicin. The suppression of K-ras expression by siRNA interfered with this synergism and inhibited both EGFR and Akt activity in A549 cells. In Ma10 cells, which harbor wild-type *EGFR* and *K-ras*, EGFR-TKIs neither suppressed Akt activity nor exhibited such synergism when combined with amrubicin. We concluded that the synergism by the combination of EGFR-TKI and amrubicin is attributable, at least partially, to *K-ras* mutation in A549 cells. The combination of EGFR-TKI and amrubicin may be a promising treatment for lung cancer with wild-type *EGFR* and *K-ras* mutation.

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Key words: Akt, amrubicin, epidermal growth factor receptor tyrosine kinase inhibitor, lung adenocarcinoma, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

Introduction

Lung cancer is the leading cause of cancer-related death worldwide. More than 80% of lung cancers are non-small cell lung carcinoma (NSCLC) with lung adenocarcinoma being the most common subtype. Over half of patients with NSCLC have advanced or metastatic disease at the time of diagnosis. Systemic chemotherapy is the standard treatment for such patients with advanced NSCLC. Despite recent improvements in diagnosis and first-line treatment, the prognosis remains poor, with an overall 5-year survival probability of only about 15% (1).

Over the last decade, the molecular heterogeneity of NSCLC has become better understood, and it is now clear that some tumors are characterized by 'driver' oncogene mutations. The most prevalent mutated oncogenes identified in lung adenocarcinoma are epidermal growth factor receptor (*EGFR*), v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*K-ras*), and anaplastic lymphoma kinase (*ALK*, where translocation and not mutation is present) (2,3). Several randomized trials demonstrated that EGFR tyrosine kinase inhibitors (EGFR-TKIs), erlotinib and gefitinib, are more effective for patients harboring activating *EGFR* mutations than standard platinum-based chemotherapy, at least in terms of response rate, progression-free survival, toxicity profile and quality of life (4-6). A multi-targeted tyrosine kinase inhibitor, crizotinib, has also been reported to exhibit clinical activity against *ALK*-translocated NSCLC (7). Although *K-ras* mutation is a major driver mutation, there is no effective treatment that targets the active form of the K-ras protein.

Several alterations in intracellular signaling are involved in the development of cancer and tumor progression (8). The phosphatidylinositol 3-kinase (PI3K)/Akt (also known as protein kinase B) pathway is believed to be a potential target cancer therapy (9). As a biological function, the proliferative and anti-apoptotic effects of Akt-mediated signaling have been

established through extensive studies (10,11). Phosphorylated Akt was detected in 70% of the tumor specimens from NSCLC patients (12), suggesting a high incidence of PI3K/Akt pathway activation in NSCLC cells. Activated Akt is also proposed to contribute to increased resistance to chemotherapy in NSCLC (13). Accordingly, if Akt also is activated in the *K-ras* mutation-harboring cancer, suppression of Akt may be a strategy for sensitizing cancer cells against chemotherapeutic agents and to improve treatment outcomes.

Amrubicin (AMR) is a totally synthetic anthracycline anticancer drug based on doxorubicin, whose hydroxyl group at position 9 is replaced by an amino group in AMR to enhance efficacy (14). In recent years, AMR monotherapy and combination therapy have been actively studied and shown promise for the treatment of small-cell lung cancer (SCLC) (15). In addition, clinical activity of AMR has been proposed for the treatment of NSCLC (16). We previously showed that Akt-suppressing agents synergistically inhibit cell growth when combined with AMR or sensitize cancer cells to AMR in SCLC cells (17). Therefore, it is hypothesized that the combination of Akt-suppressing agent and AMR can be an effective treatment strategy for NSCLC.

We report here that the combination of AMR and Akt-suppressing agents, including EGFR-TKIs, show synergistic cell growth inhibition and that this synergism by the combination of EGFR-TKIs and AMR may be involved with the *K-ras* mutation itself in A549 lung adenocarcinoma cells that have wild-type *EGFR* and mutant *K-ras* genes.

Materials and methods

Chemicals and reagents. AMR (a gift from Dainippon Sumitomo Pharama, Tokyo, Japan) and pemetrexed (PEM) (a gift from Eli Lilly, Indianapolis, IN, USA) were dissolved in distilled water and stored at -20˚C. A stock solution of cisplatin (CDDP) (a gift from Nippon Kayaku, Tokyo, Japan) was reconstituted with water, diluted in 0.9% sodium chloride solution, and stored at -20˚C. Gefitinib (a gift from AstraZeneca, Chestitre, UK), erlotinib (a gift from F. Hoffmann-La Roche, Basel, Switzerland), paclitaxel (PTX) (a gift from Brisol-Meyers-Squibb, Tokyo, Japan), 2-(4-morpholinyl)-8-phenyl-4H-l-benzopiran-4-one (LY294002) (Wako Pure Chemical Industries, Osaka, Japan), and 4',5,7-trihydroxy-isoflavone (genistein) (Wako Pure Chemical Industries) were dissolved in dimethyl-sulfoxide and stored at -20˚C. 3-(4,5-Dimethyl-thiasol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Wako Pure Chemical Industries) was dissolved in phosphate-buffered saline (PBS) and stored at -20˚C.

Cells. The sources of A549, Ma10 and PC9 cells, all of which were lung adenocarcinoma cell lines, were described previously (18). A549 and Ma10 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). PC9 cells were also maintained in RPMI-1640 medium supplemented with 10% FBS and antibiotics. These cells were grown in a humidified atmosphere of 5% CO_2 - 95% air at 37˚C.

MTT assay. MTT assay was performed to evaluate cell proliferation inhibition. Cells were counted with a hematocytometer, and 10^4 cells were incubated in 100 μl of medium containing the indicated drugs for 72 h using 96-well flat-bottom multi-plates (Nalge Nunc, Penfield, NY, USA). After 72 h, 10 μg of MTT in 10 μl PBS was added to each well and incubation was continued for an additional 4 h. Thereafter, 100 μl of 0.04 N HCl in 2-propanol was added and the multi-plates were incubated overnight to solubilize the MTT formazan crystal. The absorbance of each well was measured at a 570-nm wavelength (reference 650 nm) using a Sunrise scanning multi-well spectrometer (TECAN Japan, Kanagawa, Japan). Each experiment was performed in duplicate or triplicate for each drug concentration and was independently performed two or three times.

Flow cytometry with Annexin V. Apoptosis rates were determined by flow cytometry analysis using an Annexin V-FITC (Beckman Coulter, Fullerton, CA, USA). A549 cells were plated at a density of 10^6 cells per well in 6-well plates and then treated with LY294002 (25 μM) and/or AMR (0.1 μM) for 24 h. Staining was performed according to the manufacturer's instructions. Fluorescence-activated cell sorting (FACS) analysis was performed immediately after staining using a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

Western blot analysis. Cells were seeded in 6-well tissue culture plates. Twenty-four hours after cell seeding, cells were washed with ice-cold PBS and lysed in lysis buffer [20 mM HEPES, 10 mM EGTA (pH 8), 1% Triton X-100, 40 mM β-glyserophosphate, 2.5 mM $MgCl_2$, 2 mM Na_3VO_4] including 1 mM PMSF, 1 mM DTT, 10 mg/ml leupeptin, 20 μg/ml aprotinin, and a phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). After 5 min on ice, lysates were centrifuged at 13,000 x g for 10 min at 4˚C and the supernatant was collected. Protein was measured by using the Bio-Rad Protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA), and protein lysates containing 20 μg of total cellular protein were subjected to discontinuous SDS-polyacrylamide gel electrophoresis. Proteins were electrotransferred to a polyvinylidene fluoride (PVDF) membrane (GE Healthcare Japan, Tokyo, Japan) for 40 min at 4˚C at 200 V. Non-specific binding was blocked by incubation with 5% non-fat milk in tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature. The following primary antibodies were probed (1:100 unless otherwise indicated): anti-c-K-ras clone Ab-1 (Calbiochem, San Diego, CA, USA), anti-Akt, anti-phospho-Akt (Ser473), anti-EGFR, anti-phospho-EGFR (Y1068) (Cell Signaling Technology, Beverly, MA, USA), and anti-β-actin antibody (Sigma-Aldrich Japan, Tokyo, Japan), overnight and washed twice with TBST. After washing, proteins were detected by incubation with horseradish peroxidase-labeled secondary antibodies (GE Healthcare Japan). Finally, each protein was detected using an enhanced chemiluminescence detection system (ECL prime) (GE Healthcare Japan) and captured with an ImageQuant LAS400 (GE Healthcare Japan).

Transfection with siRNA. Either $5x10^4$ cells or $5x10^3$ cells were seeded in 6- or 96-well tissue culture plates. Twenty-four hours after cell seeding, transient small interfering RNA (siRNA) directed against specific K-ras (ON-TARGETplus siRNA SMART pool; Thermo Fischer Scientific, Rockford, IL, USA) or control siRNA-A (sc-37007; Santa Cruz Biotechnology,

Santa Cruz, CA, USA) was transfected to A549 cells according to the manufacturer's instructions. Briefly, A549 cells were treated with the indicated concentration of siRNA using 5 μl Lipofectamine 2000 transfection reagent in Opti-MEM I reduced serum medium (both from Invitrogen, Carlsbad, CA, USA) for 6 h. The medium was removed and replaced with fresh DMEM supplemented with 10% fetal calf serum and antibiotics. Cells were used 24 h after transfection for western blot analysis or MTT assay.

Assessment of combination effect. To assess the combination effect of the indicated agents qualitatively, isobologram analysis was utilized as described previously (19). The percentage of cell proliferation was calculated as: [(mean absorbance of drug-treated wells - mean absorbance of cell-free wells)/(mean absorbance of vehicle cells - mean absorbance of cell-free wells)] x 100. We used the concentration producing 50% inhibition of cell growth (IC_{50}) to evaluate dose-response interactions.

A combination index (CI) was used to compare the combination effect of the two drugs quantitatively between control and treated cells. The CI quantitatively depicts synergism (CI <1), addictive effect (CI = 1), and antagonism (CI >1). The CI for each fraction-affected value representing the percentage of proliferation inhibited by a drug was calculated using the Chou and Talalay method (20). The fraction-affected value (Fa)/CI plots were constructed in Excel 2007.

Results

Effects of LY294002 on Akt activity and interactions with chemotherapeutic agents in A549 cells. We tested the interaction between an Akt inhibitor, LY294002 and representative chemotherapeutic agents including AMR in A549 cells. A549 cells were treated with the indicated concentration of LY294002 for 1 h. LY294002 at 25 μM effectively suppressed Akt phosphorylation (Fig. 1A).

We previously reported that the combination of LY294002 and AMR synergistically inhibited the growth of N417 cells, derived from SCLC. In A549 cells, the combination of LY294002 and AMR also synergistically inhibited cell growth, whereas only additive interactions were observed in the combination of LY294002 with CDDP and PEM. In the combination of LY294002 and PTX, only antagonistic effects were observed, as judged by isobologram analysis (Fig. 1B).

To evaluate whether the synergism observed in the combination of AMR and LY294002 is attributable to an enhancement of apoptotic cell death, the binding of Annexin V to cells was measured by flow cytometry after treatment with either AMR (0.1 μM), LY294002 (25 μM) or the combination. Although Annexin V binding did not differ remarkably after treatment with the single agent compared to untreated cells, a clear increase in Annexin V binding was observed after the simultaneous combination of LY294002 and AMR (Fig. 1C).

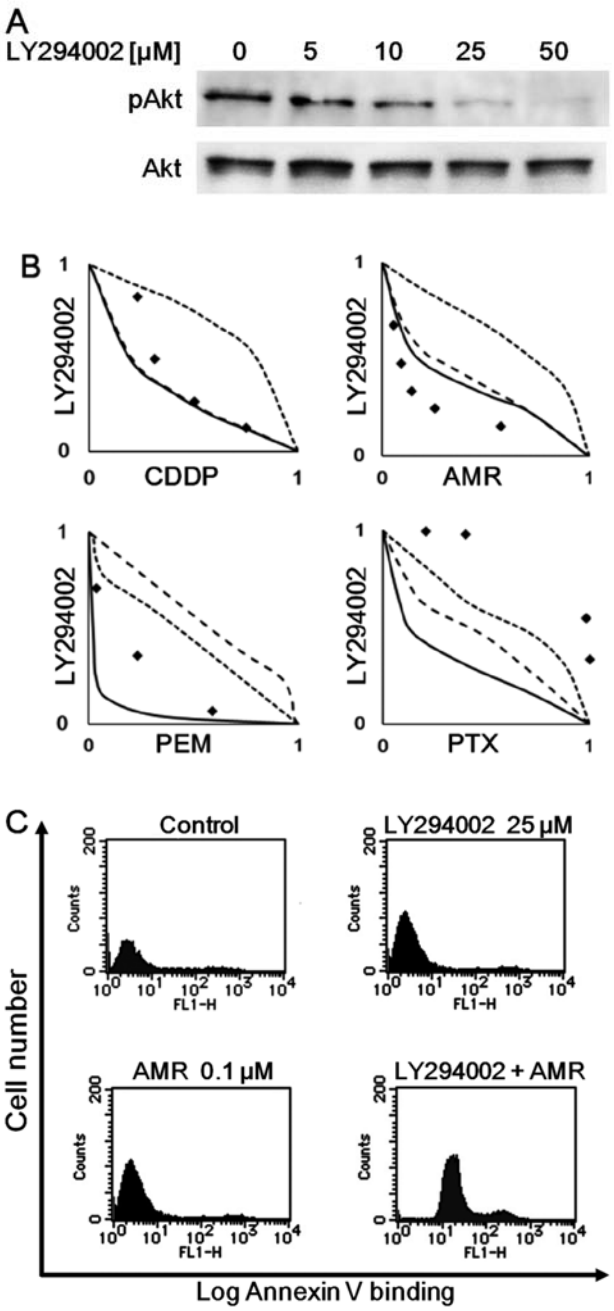

Figure 1. Combination effects of LY294002 and chemotherapeutic agents in A549 cells. (A) A549 cells were treated with the indicated concentrations of LY294002 for 1 h. Total cellular protein (20 μg) from A549 cells lysate was subjected to western blot analysis with anti-phospho-Akt (upper panel) and anti-Akt antibody (lower panel). (B) A549 cells were treated with a combination of LY294002 and cisplatin (CDDP), amrubicin (AMR), pemetrexed (PEM) or paclitaxel (PTX) for 72 h. The combination effects were evaluated by isobologram analysis. The envelopes of additivity are defined by three isoeffect lines constructed from the dose-response curves of the single agents. The concentration producing 50% cell growth inhibition (IC_{50}) of LY294002, CDDP, AMR, PEM or PTX alone is expressed as one on the ordinate and the abscissa. The plotted data points show the relative values of the concentrations producing IC_{50}. (C) A549 cells were treated with the indicated concentrations of LY294002 and/or AMR for 24 h, and the binding of Annexin V to cells was measured by a flow cytometer.

Effects of genistein on the activity of Akt and synergistic cell growth inhibition by the combination of AMR. Because Akt works downstream of tyrosine kinases (21), we tested whether genistein, a non-specific tyrosine kinase inhibitor, suppresses Akt activity and synergistically inhibits cell growth in combination with AMR. A549 cells were treated with the indicated concentration of genistein for 6 h. As expected, genistein concentration-dependently suppressed Akt activity in the

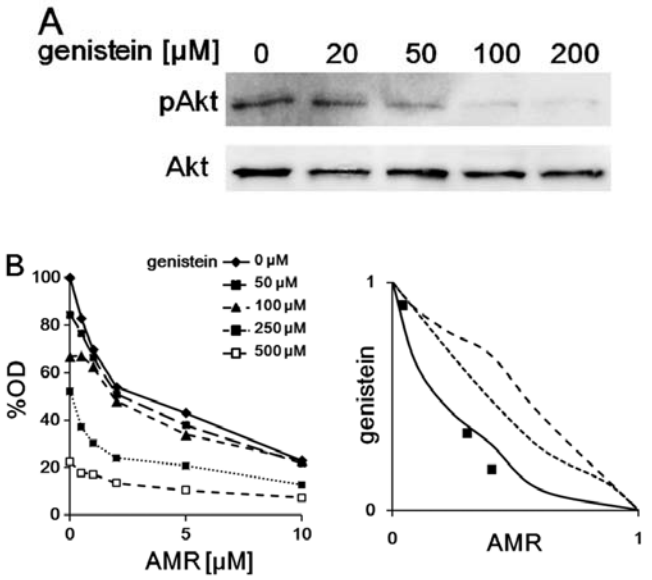

Figure 2. Effect of genistein on Akt activity and drug interaction with amrubicin (AMR) in A549 cells. (A) A549 cells were treated with the indicated concentrations of genistein for 6 h. Total cellular protein (20 μg) from the cell lysate was subjected to western blot analysis with anti-phospho-Akt (upper panel) and anti-Akt antibody (lower panel). (B) A549 cells were treated with a combination of genistein and AMR for 72 h. Dose-response curves were plotted on the basis of the data derived from the MTT assay. The survival cell fraction was expressed as the percentage of optical density (%OD) in reference to OD of the untreated cells. The combination effects were evaluated with isobologram analysis. The envelopes of additivity are defined by three isoeffect lines constructed from the dose-response curves of the single agents. The concentration producing 50% cell growth inhibition (IC_{50}) of AMR or genistein alone is expressed as one on the ordinate and the abscissa. The plotted data points show the relative values of the concentrations producing IC_{50}.

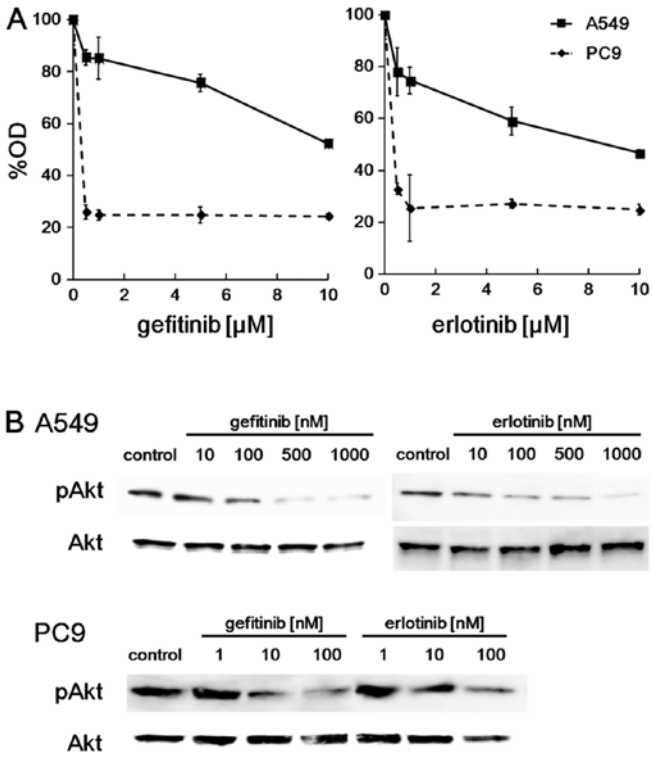

Figure 3. Effects of EGFR-TKIs on cell growth and Akt activity in A549 and PC9 cells. (A) A549 or PC9 cells were treated with various concentrations of either gefitinib or erlotinib for 72 h. The survival cell fraction was expressed as the percentage of optical density (%OD) in reference to OD of the untreated cells. Data are presented as the mean ± SD of three separate experiments. (B) A549 or PC9 cells were treated with the indicated concentrations of gefitinib or erlotinib for 2 h. The total cellular protein (20 μg) from cell lysate was subjected to western blot analysis with anti-phospho-Akt (pAkt) (upper panel) and anti-Akt (lower panel).

concentration range <200 μM (Fig. 2A). Moreover, genistein at these concentrations showed additive to synergistic growth inhibition in combination with AMR (Fig. 2B).

Effects of gefitinib and erlotinib on cell growth and Akt activity in A549 and PC9 cells. Previously, we reported that A549 and PC9 cells harbor wild-type and activating mutant (del E746-A750) *EGFR* gene, respectively (18). Using these cell lines, we evaluated the relationship between growth-inhibitory activity and Akt suppression by gefitinib or erlotinib. Both gefitinib and erlotinib demonstrated growth inhibition in both cell lines, but sensitivity to these drugs varied markedly between them. In PC9 cells with a mutated *EGFR* gene, the IC_{50}s for gefitinib and erlotinib were 10 to 100 nM. On the other hand, compared with PC9 cells, A549 cells were highly resistant in terms of growth inhibition by EGFR-TKIs with an IC_{50} of approximately 10 μM (Fig. 3A).

Akt activity in A549 and PC9 cells was evaluated after 2 h treatment with gefitinib or erlotinib. In PC9 cells, both gefitinib and erlotinib suppressed Akt activity at a concentration of 10 nM or more, indicating that the IC_{50} and the Akt-suppressing concentration of EGFR-TKIs are at similar levels. In A549 cells, on the other hand, although the IC_{50}s for gefitinib or erlotinib were approximately 10 μM, 100 nM to 1 μM of gefitinib or erlotinib suppressed Akt activity (Fig. 3B). These observations suggested that there is a discrepancy between cell growth inhibition and Akt-suppressing activity by EGFR-TKIs in A549 cells.

Synergistic cell growth inhibition by the combination of AMR and EGFR-TKIs in A549 cells. Akt-suppressing concentrations of EGFR-TKIs were apparently lower than the IC_{50} in A549 cells. It was postulated that these agents function as Akt inhibitors and demonstrated AMR-sensitizing activity in A549 cells with wild-type *EGFR*. We evaluated the combination effects of EGFR-TKIs and AMR in A549 cells. As shown in Fig. 4A, in the combination of gefitinib and AMR, three of four experimental points were plotted on the left of the predictor line of an additive effect when IC_{50} was taken as the experimental end-point (indicating a supra-additive effect). Similar results were achieved for erlotinib with AMR in A549 cells (Fig. 4B).

Effects of K-ras knockdown on the synergism and the activity of EGFR and Akt in A549 cells. In a previous study, we confirmed that A549 cells harbor oncogenic *K-ras* mutations (G12S) (18). To investigate whether the active form of K-ras is responsible for the observed synergism in the combination of EGFR-TKIs and AMR, we knocked down K-ras by siRNA. K-ras expression in A549 cells was effectively suppressed by siRNA as confirmed by immunoblot analysis (Fig. 5A). To compare the combination effects of EGFR-TKIs and AMR

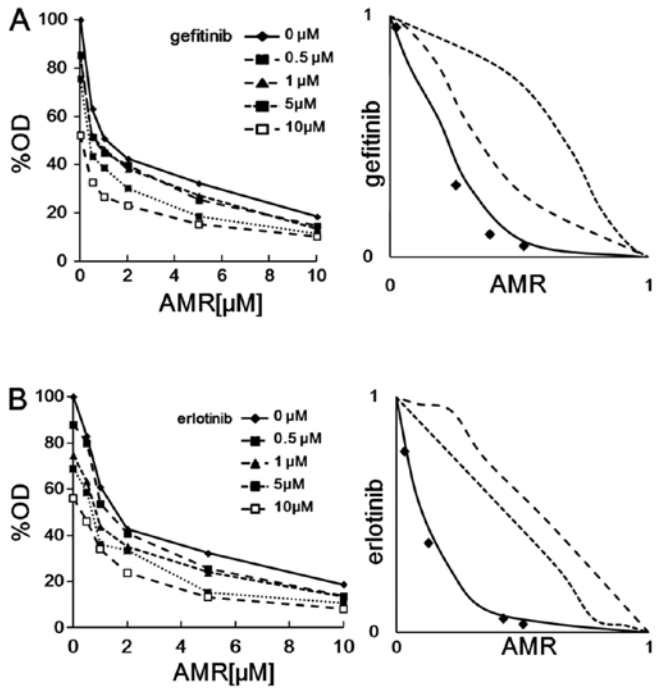

Figure 4. Combination effects of EGFR-TKIs and amrubicin (AMR) in A549 cells. A549 cells were treated with the combination of AMR and (A) gefitinib or (B) erlotinib for 72 h. Dose-response curves were plotted on the basis of the data derived from the MTT assay. The survival cell fraction was expressed as the percentage of optical density (%OD) in reference to OD of the untreated cells. The combination effects were evaluated with isobologram analysis. The envelopes of additivity are defined by three isoeffect lines constructed from the dose-response curves of the single agents. The concentration producing 50% cell growth inhibition (IC_{50}) of gefitinib, erlotinib, or AMR alone is expressed as one on the ordinate or the abscissa. The plotted data points show the relative values of the concentrations producing IC_{50}.

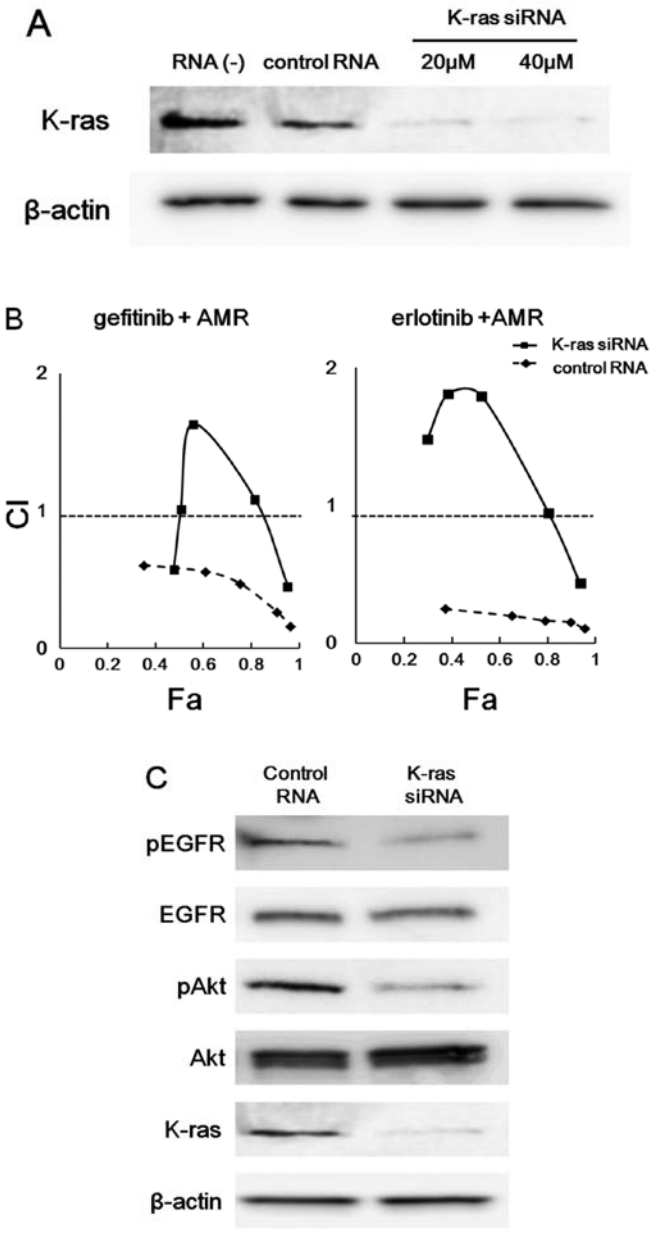

Figure 5. Effects of selective inhibition of K-ras expression on the synergism by the combination of EGFR-TKI and AMR, and EGFR and Akt activity in A549 cells. (A) A549 cells were treated with transfection reagent alone or were transfected with control siRNA or the indicated concentrations of siRNA specific for K-ras. After 24 h, the total cellular protein (20 μg) from cell lysate was subjected to western blot analysis with anti-K-ras (upper panel) and anti-β-actin (lower panel) antibody. (B) A549 cells were transfected with 20 μM of control (dotted line) or K-ras siRNA (solid line). The cells were treated with either a single agent or the combination of AMR and gefitinib or erlotinib at a fixed molar ratio (1:1) for 72 h. The viabilities of the cells were determined by the MTT assay. The combination index (CI) for each fraction-affected value (Fa) was calculated using the Chou-Talalay method. (C) A549 cells were transfected as described above. After 24 h, western blot analysis was done on A549 cell lysates with the antibodies specific for phospho-EGFR (pEGFR), phospho-Akt (pAkt), total EGFR, Akt, K-ras and anti-β-actin.

between control and K-ras knockdown cells, we performed fixed-ratio dilution experiment to calculate CI (Fig. 5B). As expected, in control cells the CI-values were constantly less than 1 for every combination of EGFR-TKIs and AMR. In K-ras knockdown cells, the curves connecting CI shifted upward. Furthermore, some of the CI values for the combination of gefitinib and AMR exceeded 1, and this tendency was remarkable in the combination of erlotinib and AMR. These results strongly suggested that oncogenic K-ras may be involved, at least partially, in the synergistic combination effect of EGFR-TKIs and AMR in A549 cells.

To investigate whether the active form of K-ras affects the EGFR-mediated signal, we evaluated EGFR and Akt activity in K-ras knockdown A549 cells. As show in Fig. 5C, both phosphorylated EGFR and Akt were decreased without a change in the total protein expression level of these proteins (Fig. 5B).

Combination effects of EGFR-TKIs and AMR in Ma10 cells with wild-type K-ras. Ma10 cells harbor wild-type *EGFR* and *K-ras* (18). We used these cells to assess the relationships among Akt activity, EGFR-TKI, and the synergism in *K-ras* wild-type cells. In Ma10 cells, both gefitinib and erlotinib could not suppress Akt activity even at the high concentration of 50 μM in spite of the clear suppression by LY294002 (Fig. 6A). The combination of LY294002 and AMR demonstrated additive to synergistic cell growth inhibition (Fig. 6B). The combination of EGFR-TKIs (either gefitinib or erlotinib) and AMR did not exert a synergistic effect (Fig. 6C), consistent with the observed synergism by the combination of an Akt-suppressing agent and AMR.

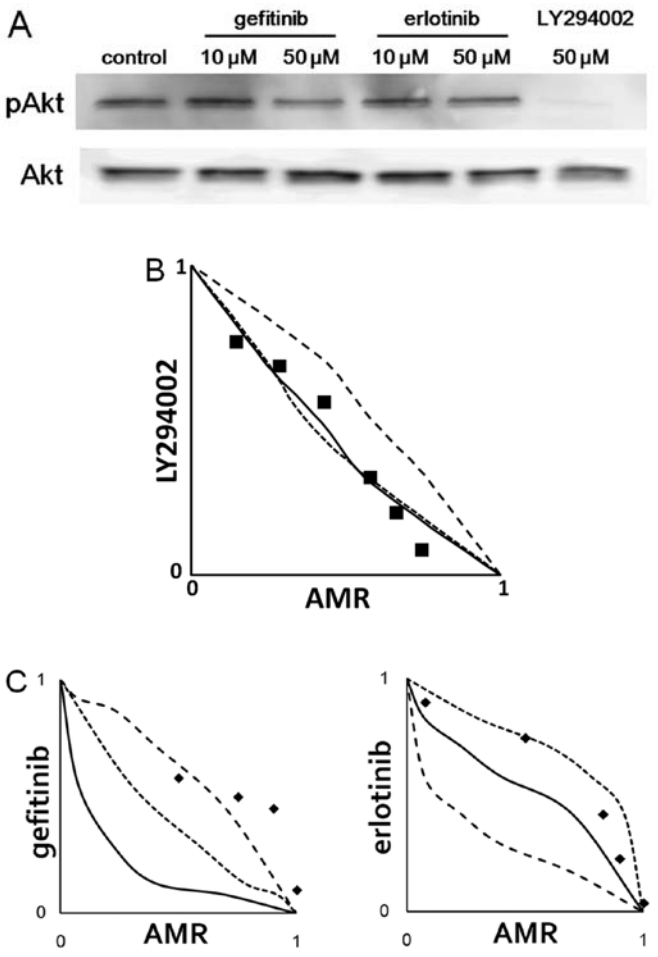

Figure 6. Effect of EGFR-TKIs on Akt activity and drug interaction with amrubicin (AMR) in Ma10 cells. (A) Ma10 cells were treated with the indicated concentrations of gefitinib, erlotinib or LY294002 for 2 h. The total cellular protein (20 μg) from cell lysate was subjected to western blot analysis with anti-phospho-Akt (pAkt) (upper panel) and anti-Akt (lower panel). (B and C) Ma10 cells were treated with a combination of LY294002 or EGFR-TKIs and AMR for 72 h. The combination effects were evaluated with isobologram analysis. The envelopes of additivity are defined by three isoeffect lines constructed from the dose-response curves of the single agents. The concentration producing 50% cell growth inhibition (IC_{50}) of LY294002, gefitinib, erlotinib, or AMR alone is expressed as one on the ordinate or the abscissa. The plotted data points show the relative values of the concentrations producing IC_{50}.

Discussion

The purpose of the present study was to clarify whether Akt-suppressing agents have therapeutic potential when combined with cytotoxic chemotherapeutic agents, including AMR, in lung adenocarcinoma without potent molecular-targeted therapy. We found that Akt-suppressing agents including clinically available EGFR-TKIs synergistically inhibit cell growth in combination with AMR. This synergism may be attributable, at least in part, to *K-ras* mutation in A549 cells.

A549 cells harbor the wild-type *EGFR* gene activating *K-ras* mutation (18). We utilized this lung adenocarcinoma cell line as a model. In A549 cells, LY294002, an Akt inhibitor, effectively suppressed Akt activity and synergistically inhibited cell growth only in the combination with AMR among the chemotherapeutic agents tested. The increase in Annexin V binding to cells after simultaneous treatment with LY294002 and AMR suggests that enhanced apoptotic cell death is a mechanism underlying this synergism. Although Akt suppression has been reported to enhance anticancer agent-induced apoptosis (22), only additive effects were observed in the combination with CDDP or PEM. Even antagonistic effects were experienced in the combination of LY294002 and PTX in this study. These observations suggest that Akt inhibition may enhance the cytotoxicity of chemotherapeutic agents in a drug-specific manner.

In general, tyrosine kinases are major upstream regulators of Akt activity, and it is expected that the suppression of tyrosine kinase activity will lead to Akt inhibition (23). On the other hand, K-ras is also proposed as a regulator of the PI3K/Akt pathway (24). Thus, we assessed whether a non-specific tyrosine kinase inhibitor, genistein, would function as well as LY294002. Similar to LY294002, genistein suppressed Akt activity and synergistically inhibited cell growth, supporting that the suppression of certain tyrosine kinases leads to Akt suppression and enhances AMR cytotoxicity even in *K-ras*-mutated A549 cells.

Recent studies have verified that lung adenocarcinoma with activating *EGFR* mutations is sensitive to EGFR-TKIs, and monotherapy with these drugs improved clinical outcomes (25,26). Conversely, in NSCLC with wild-type *EGFR*, the antitumor activity of EGFR-TKIs is limited. Indeed, judging from the IC_{50} and compared with *EGFR*-mutated PC9 cells, A549 cells with wild-type *EGFR* were 100-fold resistant to EGFR-TKIs with IC_{50}s of around 10 μM. Nevertheless, Akt activity was suppressed at concentrations ranging from 100 nM to 1 μM of EGFR-TKIs, in contrast to PC9 cells, in which the Akt-suppressing concentrations of EGFR-TKIs and the IC_{50}s were at similar levels. The discrepancy in A549 cells with respect to the Akt-suppressing concentrations and IC_{50}s may be attributable to the *K-ras* mutation, which is assumed to function as a driver mutation.

The maximum plasma concentration (C_{max}) of 225 mg/day gefitinib (250 mg/day is administered in clinical practice) is about 0.7 μM (27). C_{max} of 150 mg/day erlotinib is approximately 4 μM (28). Therefore, the Akt-suppressing concentration of EGFR-TKIs observed in A549 cells is clinically achievable.

This finding raises the possibility that Akt activity can be suppressed even though sufficient antitumor activity by EGFR-TKIs is absent in lung cancer with wild-type *EGFR*. In A549 cells, since both gefitinib and erlotinib suppressed Akt activity at clinically relevant concentrations, we evaluated the combination effect of these agents with AMR and observed synergistic cell growth inhibition. These observations support the clinical usefulness of the combination therapy by these drugs.

Since *K-ras* and *EGFR* mutations are mutually exclusive as driver mutations (2), the incidence of *K-ras* mutation should be elevated among the subgroup of lung adenocarcinoma without *EGFR* mutation. To clarify the role of *K-ras* mutation in the observed synergism, the expression of K-ras protein was suppressed by siRNA, and the combination effects of EGFR-TKIs and AMR were evaluated. Judging from the CI, the degree of synergism was decreased, and even antagonism was observed with the combination of EGFR-TKIs and AMR in K-ras knockdown A549 cells. Furthermore, in Ma10 cells, in which both the *EGFR* and *K-ras* genes are wild-type (18), only additive to antagonistic effects, and not synergistic effects, were observed in the combination EGFR-TKIs and AMR. These findings suggest that *K-ras* mutation contributes

at least partially to synergistic cell growth inhibition by the combination treatment of EGFR-TKIs and AMR.

Akt-suppressing agents consistently demonstrated synergistic effects in combination with AMR both in A549 cells in this report and in several SCLC in our previous studies (17). Furthermore, the suppression of Akt is reported to enhance the cytotoxicity of another anthracycline, doxorubicin, in other systems (29). These observations support that anthracyclines, including AMR are suitable cytotoxic drugs for combination with an Akt-suppressing agent. Actually, the combination of LY294002 and AMR exerted an additive to synergistic inhibition also in Ma10 cells.

However, neither gefitinib nor erlotinib suppressed Akt activity, and the combination of these drugs with AMR was not synergistic in Ma10 cells, although the expression level of EGFR is similar to that of A549 cells (18). Recent studies support the linkage between *K-ras* mutation and EGFR-mediated signals. Pancreatic ductal adenocarcinomas driven by *K-ras* oncogenes are dependent on EGFR signaling (30). The transfection of mutated *K-ras* to head-and-neck cancer cells induces autocrine production of EGFR ligands such as amphiregulin and transforming growth factor α, and activates the PI3K/Akt pathway (31). Activated, but not wild-type ras, facilitates nucleolin interaction with EGFR and stabilizes EGFR proteins levels, leading to synergistic anchorage-independent cell growth *in vitro* and tumor growth *in vivo* (32). In addition, the expression of constitutively active ras induces ErbB4 phosphorylation (33), and EGFR inhibitors can prevent the phosphorylation of ErbB4 (34). In the present study, we observed that the suppression of K-ras protein expression led to the inhibition of both EGFR and Akt activity in *K-ras*-mutated A549 cells, and neither gefitinib nor erlotinib suppressed Akt activity in *K-ras* wild-type Ma10 cells. Therefore, we concluded that oncogenic K-ras induces Akt activation, which can be suppressed by EGFR-TKIs in A549 cells. In addition, we propose that the synergistic effect by the combination of EGFR-TKIs and AMR may be specific in *K-ras*-mutated lung adenocarcinomas among those with wild-type *EGFR*. Further investigation is needed to clarify the precise mechanism by which active K-ras activates EGFR-mediated signaling.

The present results may be useful for considering treatments for NSCLC harboring the *K-ras* mutation. At present, molecular-targeted therapy for K-ras is not clinically available. In addition, it is reported that lung cancer with *K-ras* mutation has a poor prognosis (35). Therefore, a novel effective therapy is strongly desired for the treatment of NSCLC with *K-ras* mutation. The results of this study suggest that EGFR-TKI may function as an Akt inhibitor and enhance the cytotoxicity of AMR, at least partially, in a *K-ras* mutation-dependent manner. AMR has promising antitumor activity not only against SCLC (36), but also NSCLC (16). Therefore, we propose that the combination therapy of EGFR-TKI and AMR can be a promising therapeutic strategy for lung cancer harboring wild-type *EGFR* and activating *K-ras* mutation. Further study, including a clinical trial, is necessary to establish this combination therapy as an option for such lung cancer.

In conclusion, the combination of AMR and Akt-suppressing agents, including EGFR-TKIs, synergistically inhibits the growth of A549 cells. We propose that the combination treatment with EGFR-TKI and AMR is promising in NSCLC with wild-type *EGFR* and mutated *K-ras* genes.

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