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α-Lipoic Acid-Induced Inhibition of Proliferation and Met Phosphorylation in Human Non-Small Cell Lung Cancer Cells

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ABSTRACT

α-Lipoic acid (α-LA), a naturally occurring anti-oxidant and co-factor for metabolic enzymes, suppresses the growth of different types of tumor cells. The mechanisms that are responsible for these results, however, remain to be elucidated. In the present study, we investigated the effects of α-LA on the proliferation and activation status of definitive receptor tyrosine kinases, epidermal growth factor receptor (EGFR) and Met/hepatocyte growth factor (HGF) receptor, in gefitinib-sensitive human non-small cell lung cancer cells harboring EGFRs with an activating mutation. The enantiomers R-α-LA and S-α-LA suppressed cell proliferation and increased the level of reactive oxygen species in HCC-827 and PC-9 human non-small cell lung cancer cells in an indistinguishable dose-dependent fashion. A phospho-receptor tyrosine kinase array and cell cycle analysis indicated that α-LA decreased tyrosine phosphorylation levels of EGFR, ErbB2, and Met, and this was associated with an inhibition in the cell-cycle transition from the G_1 phase to the S phase without inducing apoptosis. Gefitinib, an inhibitor for EGFR tyrosine kinase, inhibited EGFR tyrosine phosphorylation/activation and proliferation of the cells. Instead, the addition of HGF induced Met tyrosine phosphorylation, and this was associated with a resistance to gefitinib-induced growth inhibition, which meant a gain in proliferative ability. In the presence of gefitinib and HGF, the addition of α-LA suppressed Met tyrosine phosphorylation, and this was associated with an inhibition in cell growth. These results suggest that the suppression of tyrosine phosphorylation/activation of growth factor receptors that is critical for the proliferation of human non-small cell lung cancer cells is a mechanism by which α-LA exerts growth inhibition for cancer cells.

1. Introduction

α-Lipoic acid (α-LA) is a naturally occurring co-factor for metabolic 2-oxoacid dehydrogenase multi-enzyme complexes, including pyruvate dehydrogenase complex [1,2]. α-LA has enantiomers, R-α-LA and S-α-LA, due to an asymmetric center at its C6 position. R-α-LA is a natural enantiomer, at least in bacteria [3,4]. In addition to its function as a co-factor of metabolic enzymes, α-LA has anti-oxidant and redox-regulating properties. In cells and tissues, α-LA is deprotonated and reduced to form α-lipoate and dihydro-α-lipoate, and α-lipoate and dihydro-α-lipoate exert anti-oxidant activity by reducing dehydroascorbate and glutathione disulfide to raise intracellular glutathione levels [5,6]. Because of these characteristics, α-LA has been used or proposed for the treatment of liver disease, diabetes, and ischemic tissue damage [7-10]. On the other hand, previous studies have indicated that α-LA suppresses the proliferation of different types of tumor cells such as breast cancer, hepatoma, leukemic, colon, and lung cancer cells, predominantly through facilitating apoptosis [11-19]. Different mechanisms have been proposed to account for the increase in apoptotic tumor cell death by α-LA.

Lung cancer is one of the most prevalent malignancies and is the leading cause of cancer-related deaths worldwide. Non-small cell lung cancer accounts for nearly 80% of lung cancer cases. The median survival rate for patients with metastatic non-small cell lung cancer is 8–10 months when treated with the most active combination of conventional chemotherapeutic agents [20,21]. Because growth factors and their receptor tyrosine kinases play critical roles in not only growth and apoptosis but also in the malignant behaviors of cancer, including invasion, metastasis, and drug resistance. Therefore, growth factors and receptors have gained much attention as potential molecular targets in anticancer therapy. Among receptor tyrosine kinases, the receptor for epidermal growth factor (EGF) has been an attractive target for the development of therapeutic agents, because it is expressed in a majority of non-small cell lung cancer cases. Indeed, the small-molecule EGF receptor tyrosine kinase inhibitors, including gefitinib and erlotinib, have been used for the treatment of patients with non-small cell lung cancer [22]. In particular, non-small cell lung cancer cells that harbor EGF receptors with an activating mutation have been associated with a favorable response to gefitinib and erlotinib [23]. However, even patients who show a dramatic response to initial treatment with gefitinib or erlotinib develop acquired resistance almost without exception after varying periods of time [23]. Among the different mechanisms by

which non-small cell lung cancer cells acquire resistance to gefitinib and erlotinib, recent studies have shown the activation of Met tyrosine kinase, the receptor for hepatocyte growth factor (HGF) is associated with resistance to gefitinib and erlotinib [24,25]. Taken together, the proliferation and survival of non-small cell lung cancer cells are likely to be particularly dependent on the activation of signaling pathways that are triggered by EGF and Met receptor tyrosine kinases.

In the present study, we investigated the effect of α-LA on the proliferation of gefitinib-sensitive human non-small cell lung cancer cells harboring an EGF receptor with an activating mutation. We found that α-LA inhibited the phosphorylation/activation of Met and EGF receptor tyrosine kinases, and this was associated with growth inhibition and suppression of HGF-induced resistance to gefitinib in non-small cell lung cancer cells.

2. Materials and Methods

2.1. Cell culture and evaluation of cell proliferation

R-α-lipoic and S-α-lipoic acids were kindly provided by Dr. Tetsuya Konishi (Niigata University of Pharmacy and Applied Life Sciences), as originally prepared by GeroNova Research, Inc. (Richmond, CA). Gefitinib-sensitive human lung adenocarcinoma cell lines, PC-9 and HCC827, with an EGF receptor mutation were purchased from Immuno-Biological Laboratories Co (Takasaki, Gunma, Japan) and from the American Type Culture Collection (Manassas, VA), respectively. The cells were cultured in RPMI1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 units/ml streptomycin, and 2 mM glutamine. To measure the cell proliferation, the cells were cultured on 6-well cell culture plates at a cell density of 1.0×10^5 / well (HCC-827) or 0.5×10^5 / well (PC-9) for 24 hours. The medium was changed, reagents were added, and the cells were cultured for 2 or 3 days. After washing with PBS, the cells were dissociated, and the number of cells was measured by hemocytommeter. Data are shown as the mean +/- SD.

2.2. Western blotting

Cells were lysed in cell lysis buffer containing phosphatase inhibitor cocktail and proteinase inhibitor cocktail (Sigma), and the protein concentrations were determined using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Aliquots of 500 μg of total proteins were immunoprecipitated with the appropriate antibodies; and, immune complexes were recovered with Protein G-Sepharose beads (Zymed Laboratories, South San Francisco, CA). For Western blotting analysis, immunoprecipitates or 40 mg of total protein were resolved by SDS-polyacrylamide gel electrophoresis, and the proteins were then transferred onto PVDF membranes (Bio-Rad). After washing 4 times, membranes were incubated with 5% skimmed milk for 1 hour at room temperature, and then incubated overnight at 4°C with the following primary antibodies: anti-Met (25H2); anti-phospho-Met (Y1234/Y1235) (D26); anti-human EGFR (C74B9); anti-phospho-EGFR (Y1068); anti-Akt, or phospho-Akt (Ser473); and anti-human/mouse/rat ERK1/ERK2, or anti-phospho ERK1/ERK2 (T202/Y204) antibodies (1:1000 dilution, Cell Signaling Technology, Beverly, MA). After washing 3 times, membranes were incubated for 1 hour at room temperature with species-specific horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized using SuperSignal West Dura Substrate (Thermo Fisher Scientific Inc.,

Rockford, IL). The membrane was developed with ECL detection reagent (ImmunoStar, Wako Pure Chemical, Osaka, Japan), and the images were obtained using an LAS-3000 (Fuji Film, Tokyo, Japan). Each experiment was performed at least 3 times independently.

2.3. Analysis of phosphorylated receptor tyrosine kinases

The cells on each culture plate were washed twice with chilled PBS, and scraped using a chilled lysis buffer that was composed of 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 1% NP-40, 1% Triton, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 10 mg/mL aprotinin, 10 mg/mL leupeptin, and 10 mg/mL pepstatin. The cells in the tubes were rotated at 4 °C for 30 min, and the cell lysate was prepared after centrifugation. The protein concentration of the lysate was determined using a BCA Protein Assay Kit. The cell lysate was applied onto the array membrane provided by the human p-RTK array kit (R&D Systems), and detection of tyrosine phosphorylation of receptor tyrosine kinases was performed according to the manufacturer's protocol. In brief, the array membrane was incubated with horseradish peroxidase-conjugated anti-phospho-tyrosine antibody for 2 hours at room temperature. The membrane was developed with ECL detection reagent (ImmunoStar), and the images were obtained using an LAS-3000 (Fuji Film, Tokyo, Japan).

2.4. Cell-cycle analysis

Cells were cultured in either the absence or the presence of 2 mM α-LA for 12 and 24 hours, and then were subjected to cell-cycle analysis. The proportion of cells at different phases of the cell cycle was monitored by flow cytometer using a Cell Cycle Phase Determination Kit, according to the manufacturer's instructions (Cayman Chemicals, Ann Arbor, MI). Briefly, cells were collected and fixed in 70% ethanol overnight at 4 °C, and stained with propidium iodide. A total of 20,000 cells per sample were collected and analyzed using a flow cytometer (FACSCanto II, Becton-Dickinson, San Jose, CA), at an excitation wavelength of 488 nm and at an emission wavelength of 630 nm. Doublet cells and cell debris were identified and discarded, as previously described [26]. The cell cycle distribution was calculated by using CellQuest software (Becton Dickinson Immunocytometry System, San Jose, CA, USA).

2.5. Measurment of reactive oxygen species

The level of reactive oxygen species (ROS) was measured by a converting reaction of 2’,7’-dichlorodihydrofluorescin diacetate to 2’,7’-dichlorodihydrofluorescein, using an OxiSelect Intracellular ROS Assay Kit (Cell Biolabs, Inc), according to the manufacturer’s instructions. Briefly, HCC-827 cells were seeded at 20,000 cells / well of 96-well black plates (Greiner) and cultured with RPMI1640 medium supplemented with 10% fetal bovine serum for 24 hours. The cells were washed three times with PBS, and were loaded with 0.1 X 2’,7’-dichlorodihydrofluorescin diacetate in PBS for 1 hour at 37 ℃ in a CO_2 incubator. The cells were washed three times with PBS and cultured with or without 2 mM R-α-/S-α-LA (1/1), R-α-LA, or S-α-LA in PBS at 37 ℃ in a CO_2 incubator. ROS activities were measured by ARVO MX (PerkinElmer) as relative fluorescence units (RFU).

2.6. Statistical analysis

We used a Student’s *t*-test to determine the statistical significance. $p < 0.05$ was considered significant.

3. Results

3.1. Growth inhibition

To examine the effect of lipoic acid on the proliferation of human lung cancer cells, human non-small cell lung cancer cell lines, HCC-827 and PC-9, were cultured for 2 days in either the absence or presence of α-LA (Fig. 1A). The number of HCC-827 cells increased 3.1-fold during 2 days. α-LA dose-dependently suppressed the proliferation of cells, and the number of cells remained at the initial level in the presence of 2.0 mM α-LA. A similar growth inhibitory effect by α-LA was seen in PC-9 cells. The concentration of α-LA that was required for a 50% growth inhibition ranged from 0.25 - 0.5 mM.

α-LA is composed of the optic isomers R-α-LA and S-α-LA, but only R-α-LA is a natural product. R-α-LA, but not S-α-LA, plays the role of a co-enzyme in metabolic regulation, while both R- and S-α-LAs have anti-oxidant action. To investigate whether the co-enzyme function of α-LA participates in its growth inhibitory action, the effects of chemically synthesized R-α-LA and S-α-LA on the proliferation of HCC-827 were examined (Fig. 1B). R-α-LA, S-α-LA, and an equimolar mixture of R- and S-α-LAs inhibited the proliferation of these lung cancer cell lines in an indistinguishable dose-dependency. Approximately the same results were obtained when PC-9 cells were used (not shown). When the change in cellular ROS levels was measured in HCC-827 cells, R-α-LA and S-α-LA, and an equimolar mixture of R- and S-α-LAs enhanced cellular ROS levels in an indistinguishable dose-dependency (Fig. 1C). These results suggest that the biochemical function of α-LA as a co-factor for the enzyme reaction may not be involved in growth inhibition in human lung cancer cells.

3.2. Change in cell-cycle transition

The above results suggested that α-LA might inhibit the cell-cycle transition and/or it might facilitate apoptosis. Therefore, we analyzed the change within the cell-cycle transition in HCC-827 cells that was influenced by α-LA (Fig. 2). The cells that had been pre-cultured for 24 hours were subjected to treatment with α-LA, and the cell-cycle distribution was analyzed at 12 and 24 hours. In control cells cultured without α-LA, the cells in the G1 and S phases were 41.8 and 30.6%, respectively, at 12 hours, and they were 49.6 and 27.0%, respectively, at 24 hours. In cells cultured with α-LA, the cells in the G_1 and S phases were 56.0 and 20.3%, respectively, at 12 hours and 66.4 and 14.8%, respectively, at 24 hours, indicating a clear increase in the G_1 population. These results indicate that α-LA inhibited the

cell-cycle transition from the G_1 phase to the S phase.

In cell-cycle analysis via a flow cytometer, the apoptotic cells increased by α-LA were detectable in the hypodiploid population, which is a lower fluorescent cell population [12,13]. However, α-LA did not increase the apoptosis of HCC-827 non-small cell lung cancer cells, at least in our experimental conditions.

3.3. Change in growth factor receptor activation

Growth factors play a definitive role in the proliferation and survival of cells. Therefore, to investigate the mechanism by which α-LA might exhibit its growth inhibitory action, we analyzed changes in the phosphorylation/activation status of receptors for growth factors that could be caused by α-LA. HCC-827 cells were either untreated or treated with α-LA for 12 hours and the phosphorylation status of growth factor receptors was analyzed via the phosphorylated receptor tyrosine kinase array (Fig. 3). In cells cultured without α-LA, tyrosine phosphorylation was strongly detectable in receptors for the epidermal growth factor (EGF) family, in EGF receptors, in ErbB2 receptors, and in Met receptors for hepatocyte growth factor (HGF). The results suggested that both the proliferation and the survival of human lung cancer cells are predominantly promoted by the activation of receptors for EGF, ErbB2, and Met. It is important to note that Met tyrosine phosphorylation was mostly undetectable in cells treated with α-LA, and that α-LA significantly decreased the tyrosine phosphorylation of receptors for EGF and ErbB2.

Because the tyrosine phosphorylation of receptors for Met/HGF and EGF was strong, and Met tyrosine phosphorylation was mostly diminished upon stimulation with α-LA in the receptor tyrosine kinase array, we used Western blot analysis (Fig. 4) to detect the changes in the phosphorylation of these receptors and typical signaling molecules that are involved in cell proliferation and survival. In control culture of HCC-827 cells, tyrosine phosphorylation of Met was clearly detectable, whereas it was strongly suppressed in the cells treated with 2.0 mM α-LA, at 1, 12 and 24 hours (Fig. 4A). Tyrosine phosphorylation of EGF receptor in cells treated with α-LA was slightly lower than that seen in the control culture. The phosphorylation of Akt was not significantly changed, whereas Erk1/2 phosphorylation was lower in α-LA-treated cells at 12 and 24 hours, compared with the control cells. When the cells were treated with varying concentrations of α-LA for 24 hours, Met tyrosine phosphorylation was strongly suppressed by α-LA in a dose-dependent manner (Fig. 4B). Tyrosine phosphorylation of EGF receptor was also suppressed by α-LA, whereas the

suppression was less, compared with that seen in Met receptor. The phosphorylation of Akt was unchanged, whereas Erk1/2 phosphorylation was decreased by α-LA.

3.4. α-LA and gefitinib-resistance by HGF

Gefitinib is an inhibitor of the tyrosine kinase in EGF receptor, and it is effective in the treatment of patients with non-small cell lung cancer cells that possess an EGF receptor with an activating mutation [22]. HCC-827 and PC-9 cells express EGF receptors with an activating mutation. Recent studies have indicated that activation of the HGF-Met pathway is associated with resistance against gefitinib in human non-small cell lung cancer cells [24]. Therefore, we tested whether α-LA might influence the HGF-dependent resistance of non-small lung cancer cells for gefitinib.

When HCC-827 cells were cultured in the presence of gefitinib +/- HGF for 3 days, the growth inhibition by gefitinib was cancelled by 50 ng/mL HGF (Fig. 5A). That result indicates that the activation of Met by HGF confers a drug resistance against gefitinib. α-LA inhibited the proliferation of cells in the presence of gefitinib and HGF (Fig. 5A), indicating that α-LA largely abrogated HGF-induced resistance to gefitinib. Analysis of the phosphorylation status of Met, EGF receptor, Akt and Erk1/2 indicated that tyrosine phosphorylation of EGF receptor, and serine phosphorylation of Akt and Erk1/2 were strong in the control culture (Fig. 5B). Gefitinib inhibited EGF receptor phosphorylation to undetectable levels, and this was associated with an inhibition in Akt and Erk1/2 phosphorylation. Those results indicated that Akt and Erk1/2 phosphorylation depended on EGF receptor activation. The addition of HGF induced Met tyrosine phosphorylation, and this was associated with the phosphorylation of Akt and Erk1/2, indicating that activation of Akt and Erk1/2 depends on Met activation. When the cells were cultured in the presence of gefitinib, HGF, and varying concentrations of α-LA for 24 hours, HGF-dependent Met tyrosine phosphorylation was dose-dependently inhibited by α-LA. Under these conditions, Erk1/2 phosphorylation, but not Akt phosphorylation, was suppressed by α-LA.

4. Discussion

In the present study, using human non-small cell lung cancer cells in culture, we found the following results: 1) α-LA suppressed the proliferation of human non-small cell lung cancer cells via the inhibition of the cell-cycle transition from the G1 phase to the S phase; 2) the inhibitory effect of enantiomers of α-LA, R-α-LA and S-α-LA on cell proliferation were indistinguishable; 3) various receptor tyrosine kinases, EGFR, ErbB2, and Met, were tyrosine phosphorylated, and α-LA suppressed the tyrosine phosphorylation of these receptors; and, 4) HGF induced resistance to the growth inhibitory action of gefitinib, whereas α-LA largely suppressed HGF-induced Met tyrosine phosphorylation, and this was associated with the suppression of the HGF-dependent resistance to gefitinib.

α-LA has enantiomers, R-α-LA and S-α-LA, and the major form of naturally occurring α-LA is R-α-LA. The kineticα-LAnalysis of a partial enzymatic reaction of pyruvate dehydrogenase complex indicated that dihydrolipoamide dehydrogenase has a clear preference for R-α-LA, and it catalyzed R-α-LA 24-times faster when compared with S-α-LA [27]. S-α-LA inhibited the R-α-LA-dependent enzyme reaction with an inhibition constant that was similar to its Michaelis constant [27]. In contrast, no significant difference was seen between R-α-LA and S-α-LA in their inhibitory action on the proliferation of human non-small cell lung carcinoma cells. It is likely that the biological function of α-LA—as a cofactor of 2-oxoacid dehydrogenase multi-enzyme complexes—does not participate in the growth inhibitory action on human non-small cell lung carcinoma cells.

The proliferation and survival of human non-small cell lung carcinoma cells significantly depends on the activation of tyrosine kinase receptors, EGFR, ErbB2, ErbB3, and Met [28]. In our study, the tyrosine phosphorylation of EGFR, ErbB2, and Met was seen, whereas tyrosine phosphorylation/activation was not seen in other receptor tyrosine kinases, neither by tyrosine phosphorylation array nor by Western blot analysis. Thus, the proliferation and survival of HCC-827 and PC-9 cells used in the present study seemed to depend on the activation of EGFR, ErbB2, and Met. We found that α-LA suppressed the tyrosine phosphorylation of EGFR, ErbB2, and Met. We considered that the suppression of the tyrosine phosphorylation/activation of EGFR, ErbB2, and Met by α-LA is a mechanism by which α-LA inhibits the proliferation of human non-small cell lung carcinoma cells. Erk1/2 and PI3K-Akt pathways participate as representative intracellular signaling molecules in the proliferation and survival of a wide variety of cells. Activation of Erk1/2 and/or PI3K-Akt

pathways are involved in the promotion of cell proliferation and survival that is mediated by EGFR and Met [29]. α-LA showed no clear effect on Akt phosphorylation, while α-LA attenuated Erk1/2 phosphorylation. In the present study, the suppression of the tyrosine phosphorylation of EGFR, ErbB2, and Met might have led to the attenuation of Erk1/2 phosphorylation, which we associated with the growth inhibition of HCC-827 and PC-9 cells by α-LA. However, the suppressive effect of α-LA on Erk1/2 phosphorylation was not remarkable, when compared with the suppressive effect on Met tyrosine phosphorylation. Perhaps intracellular signaling pathways other than Erk1/2 and PI3K-Akt may possibly be involved in the growth inhibition by α-LA.

Gefitinib and erlotinib, selective inhibitors for EGFR tyrosine kinase, have shown a therapeutic effect on non-small cell lung cancer cells harboring EGF receptor with an activating mutation [23,30]. The therapeutic effect of these EGFR tyrosine kinase inhibitors indicates that the proliferation and survival of human non-small cell lung cancer cells addictively depends on the activation of EGFR. HCC-827 and PC-9 cells have an activating mutation in their EGFR, and, therefore, these cells are sensitive to gefitinib. Human non-small cell lung cancer cells exposed to gefitinib for longer than a certain period of time will acquire a resistance to the growth inhibitory action of gefitinib. Different mechanisms by which non-small cell lung cancer cells acquire resistance to gefitinib have been elucidated [30], and include the following: 1) a second mutation in EGFR [22,31]; 2) activation of Met by amplification of the *met* gene [24]; and, 3) activation of Met by the overexpression of HGF [25,32]. Thus, the activation of Met is tightly associated with resistance to gefitinib. Consistently, the proliferation of HCC-827 cells was inhibited by gefitinib, whereas the addition of HGF significantly reversed proliferation even in the presence of gefitinib, which indicated that HCC-827 cells acquired resistance to gefitinib through activation of the HGF-Met pathway. α-LA dose-dependently suppressed the proliferation of HCC-827 cells recovered by HGF in the presence of gefitinib, indicating a recovery of the sensitivity to gefitinib. HGF induced Met tyrosine phosphorylation/activation in the presence of gefitinib, but α-LA inhibited Met activation induced by HGF. These results clearly indicate that α-LA recovers the sensitivity to gefitinib through the suppression of HGF-induced Met tyrosine phosphorylation/activation.

Previous studies have demonstrated that α-LA facilitates the apoptosis of different types of human tumor cells with different mechanisms [11-19]. In MCF-7 breast cancer cells, SMMC-7721 hepatoma cells, and HT-29 colon cancer cells, α-LA decreased ROS, and this

was associated with an increase in apoptosis [12,15,17]. By contrast, in FaO and HepG2 hepatoma cells and in H460 lung cancer cells, α-LA enhanced ROS, which was associated with an increase in apoptosis [16,19]. Therefore, there were discrepancies in the effect of α-LA on ROS, perhaps due to differences in tumor cell types and/or culture conditions. In the present study, α-LA inhibited the cell-cycle transition from the G1 phase to the S phase, while we obtained no clear data showing the induction of apoptosis by α-LA. Because the activation of EGFR, ErbB2, and Met is primarily required for the proliferation of human non-small lung cancer cells, we assumed that the inhibition of cell proliferation by α-LA was attributable to the suppression of EGFR, ErbB2, and Met tyrosine phosphorylation.

We unexpectedly found that rather than facilitating apoptosis in human non-small cell lung cancer cells, α-LA inhibits the activation of growth factor receptors, particularly Met, and this was associated with an inhibition of the cell-cycle transition and of cell proliferation. Because the activation of receptors for EGF and Met plays a definitive role in the proliferation of non-small cell lung cancer cells, the inhibitory effect on the activation of these growth factor receptors might be the primary mechanism whereby α-LA inhibits the proliferation of cells, although this mechanism remains to be addressed. On the other hand, α-LA has been used as a medicine for patients with diabetes and diabetic neuropathy, and is often taken as a dietary supplement because of its anti-oxidant action. We cannot exclude the possibility that α-LA might influence tumor growth when taken as a dietary supplement or medicine. Nevertheless, considering the relatively short half-life of α-LA in the blood, as well as pertinent safety issues, the combination of α-LA with an effective delivery technique to tumor region would be required, if anti-tumor action of α-LA could be expected in clinical application.

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Disclosure of Potential Conflicts of Interest:

No potential conflicts of interest were disclosed by the authors.

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Figure Legends

Fig. 1. Effects of α-LA on the cell proliferation and ROS activity in human non-small cell lung cancer cells. (A) Effect of α-LA on HCC-827 and PC-9 cells. (B) Effect of α-LA enantiomers, R-α-LA and S-α-LA on HCC-827 cells. The cells were cultured in either the absence or presence of α-LA for 2 days, and the number of cells was determined. Data are shown as the mean +/- S.D. ($n = 3$ in each group). (C) Change in ROS activity by α-LA enantiomers, R-α-LA and S-α-LA in HCC-827 cells. Data are shown as the mean +/- S.D. The differences between values as indicated by asterisks are versus the PBS control and are statistically significant (*, $p < 0.01$).

Fig. 2. Change in the cell-cycle distribution of HCC-827 human non-small cell lung cancer cells by α-LA. The cells were cultured in either the absence or presence of 2.0 mM α-LA for 12 and 24 hours, and were subjected to cell-cycle analysis, using a flow cytometer.

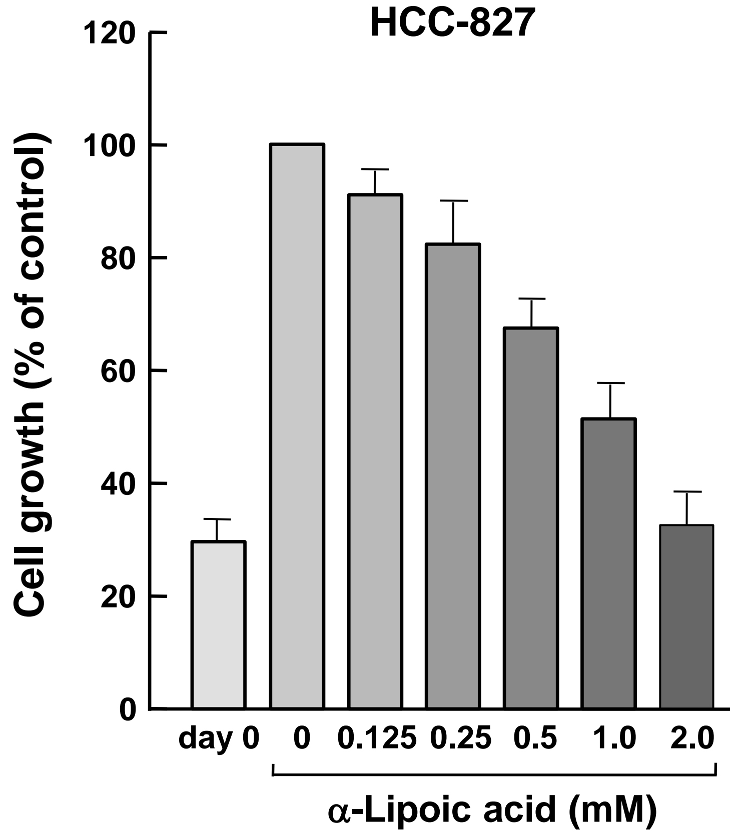
Fig. 3. Changes in tyrosine phosphorylation status of receptor tyrosine kinases by α-LA in HCC-827 cells. The cells were either untreated or treated with α-LA for 12 hours, and the phosphorylation status of growth factor receptors was analyzed via phosphorylated receptor tyrosine kinase array.

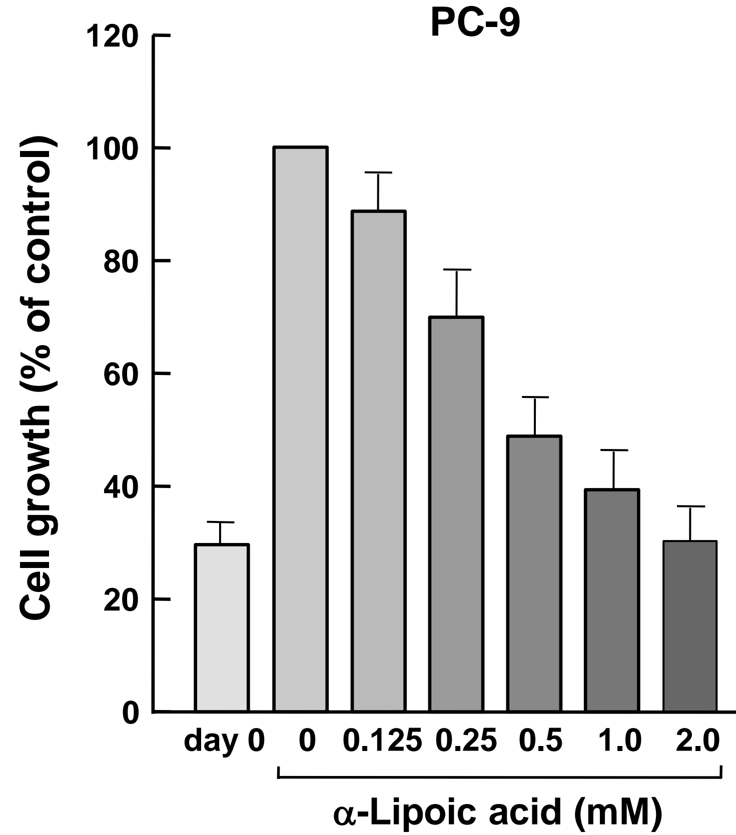
Fig. 4. Effects of α-LA on the phosphorylation of Met, EGF receptor, Erk1/2, and Akt in HCC-827 cells. Phosphorylation status of Met receptor, EGF receptor, Erk1/2, and Akt was analyzed at 1, 12, and 24 hours following the addition of 2.0 mM α-LA (A), or they were analyzed at 24 hours following the addition of varying concentrations of α-LA (B). The phosphorylation and expression of Met receptor, EGF receptor, Erk1/2, and Akt were analyzed by Western blot. In (B), the cells were cultured for 24 hours.

Fig. 5. Effects of gefitinib, HGF, and α-LA on the proliferation and phosphorylation of signaling molecules in HCC-827 cells. (A) Changes in cell proliferation either untreated or treated with a combination of 1 mM gefitinib, 50 ng/mL HGF, and varying concentrations of α-LA. The cells were cultured for 3 days, and the number of cells was determined. (B) Changes in the phosphorylation of receptors for Met and EGF and that of Erk1/2 and Akt. Cells were either untreated or treated for 1 hour with 1 mM gefitinib and/or 50 ng/mL HGF.

(C) Changes in the phosphorylation status of Met receptor, EGF receptor, Erk1/2, and Akt by varying concentrations of α-LA in cells cultured with gefitinib +/- HGF. The cells were cultured for 24 hours.

Michikoshi et al, Fig. 1A





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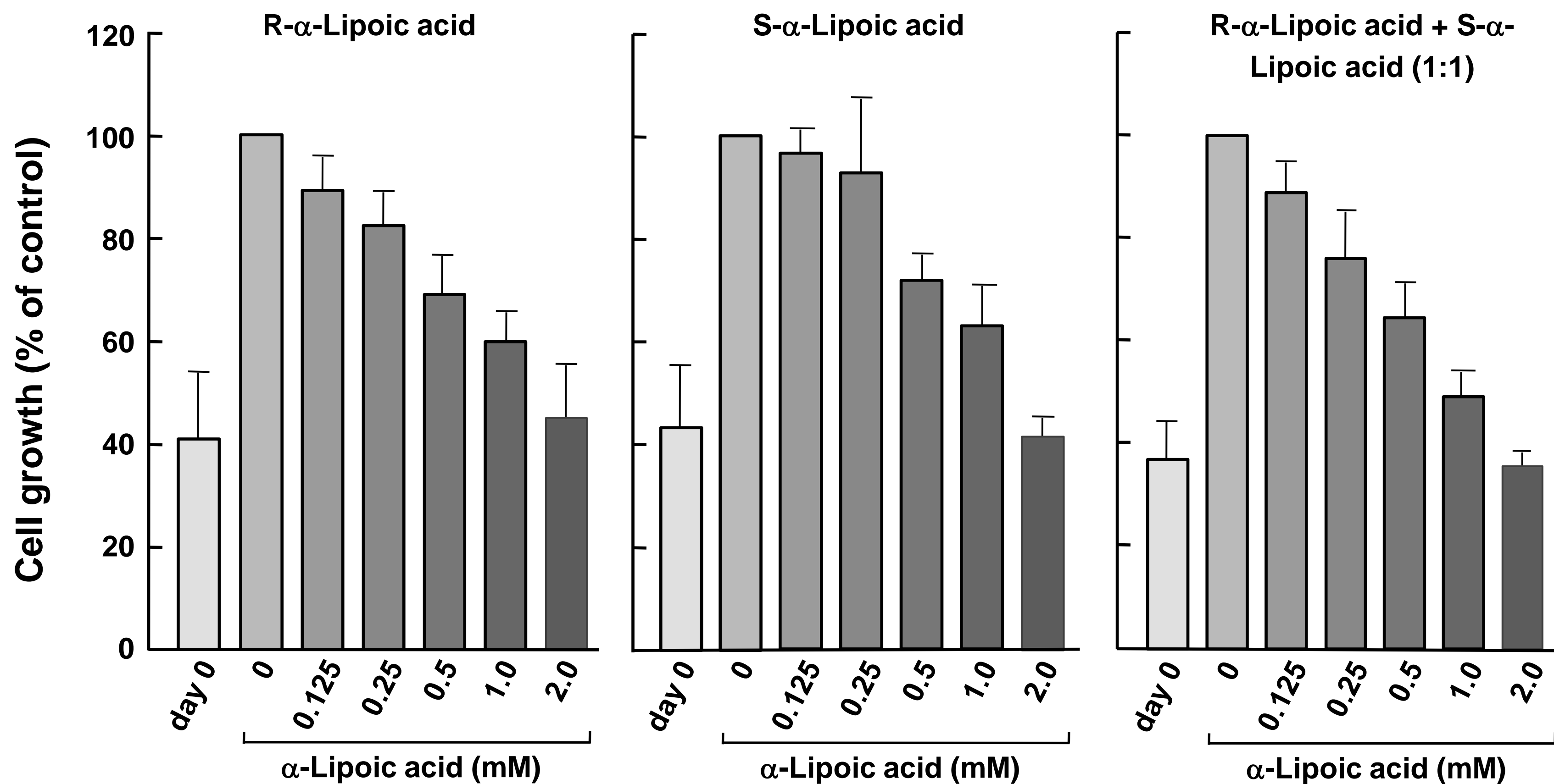

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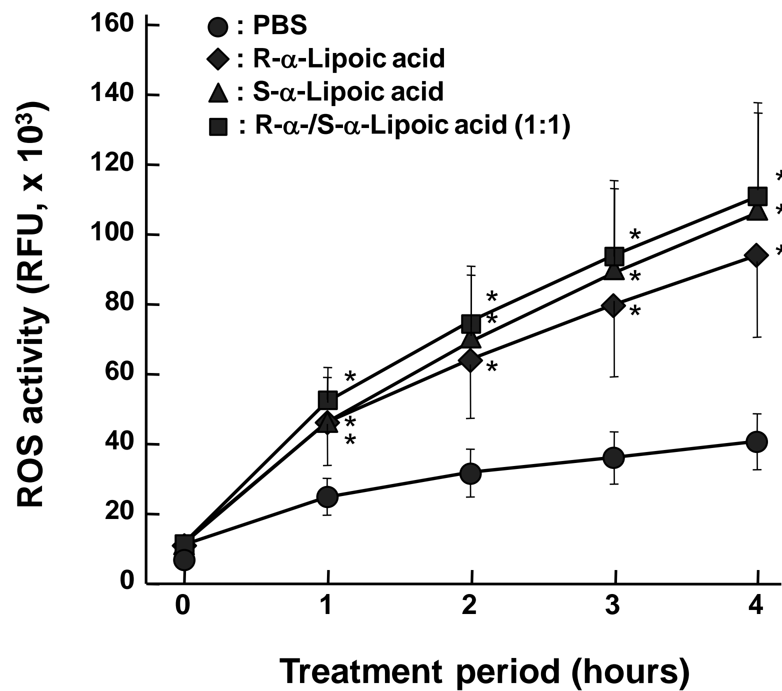

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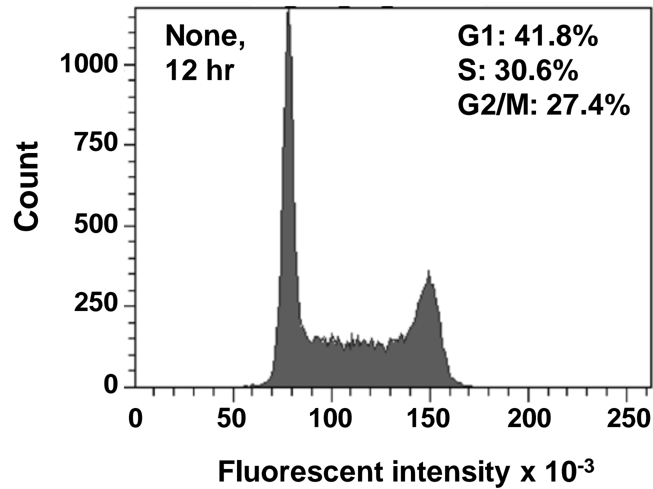
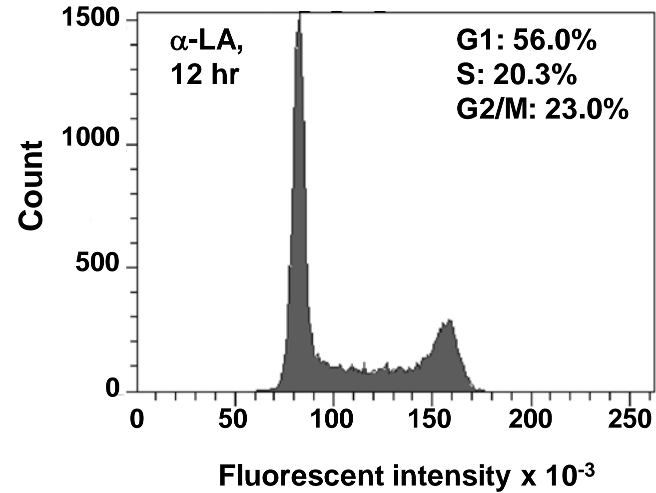
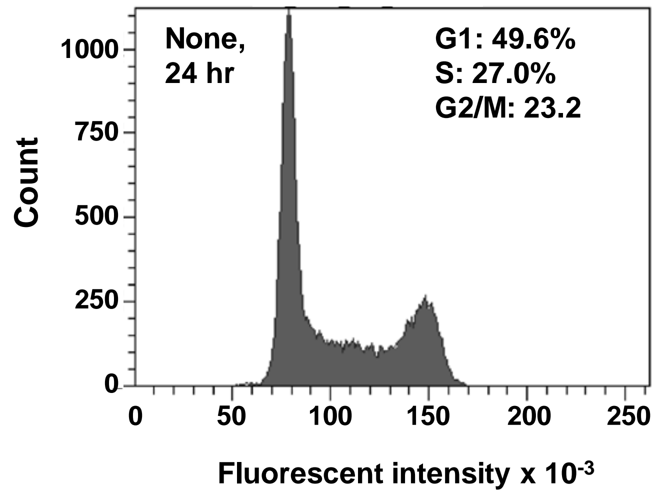
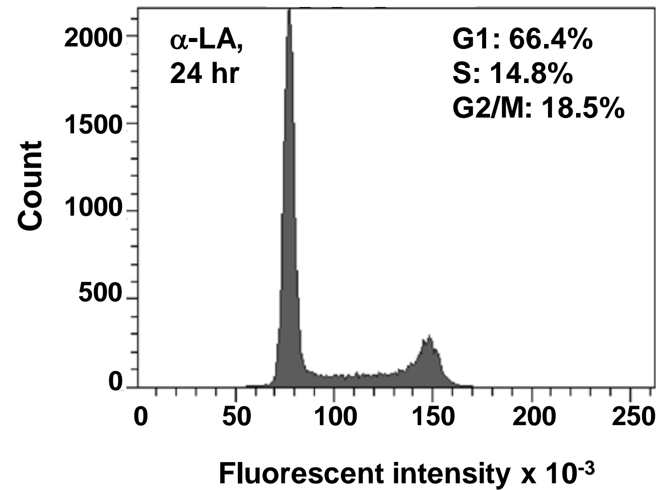
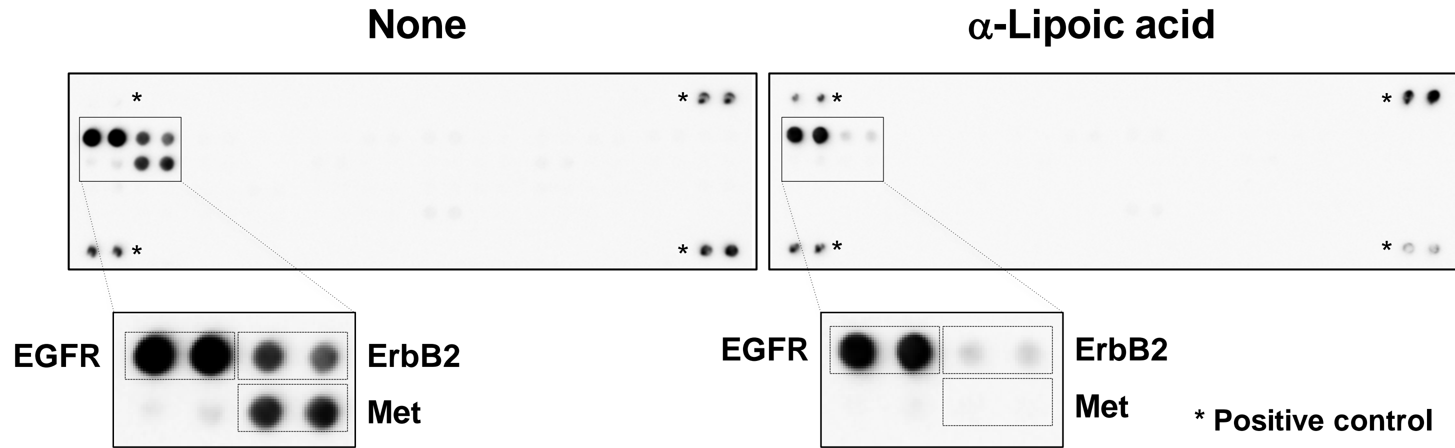


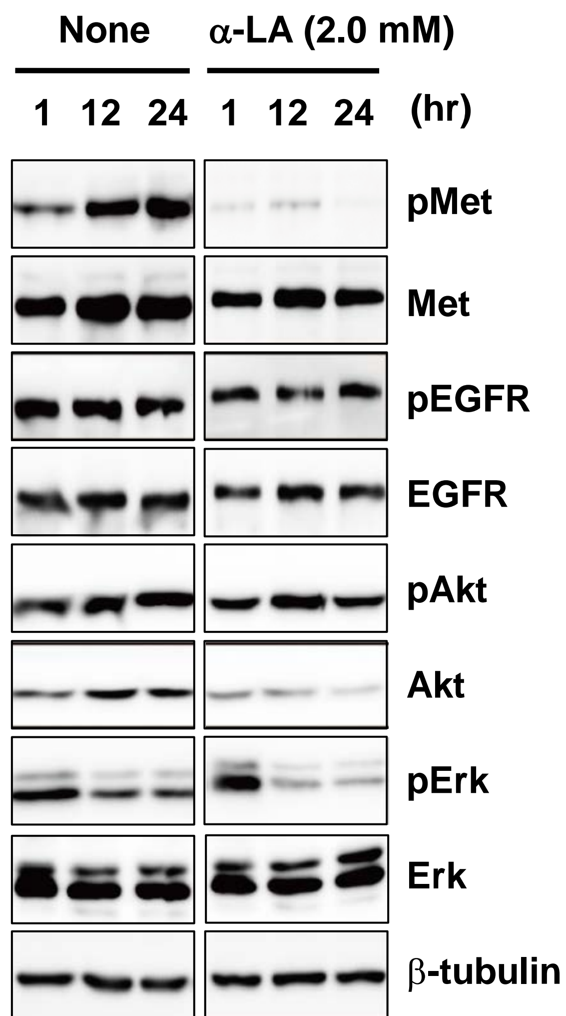


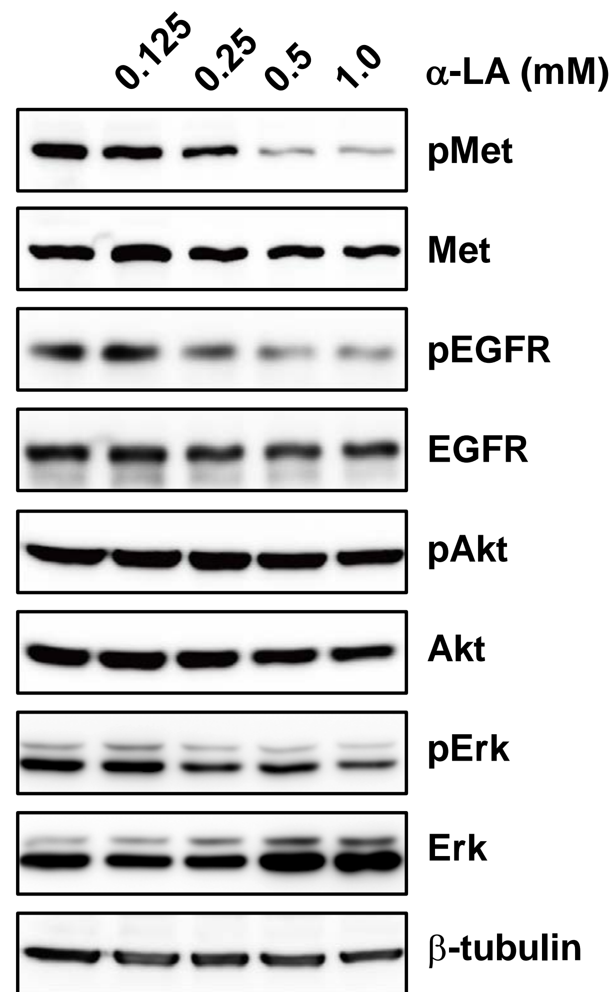


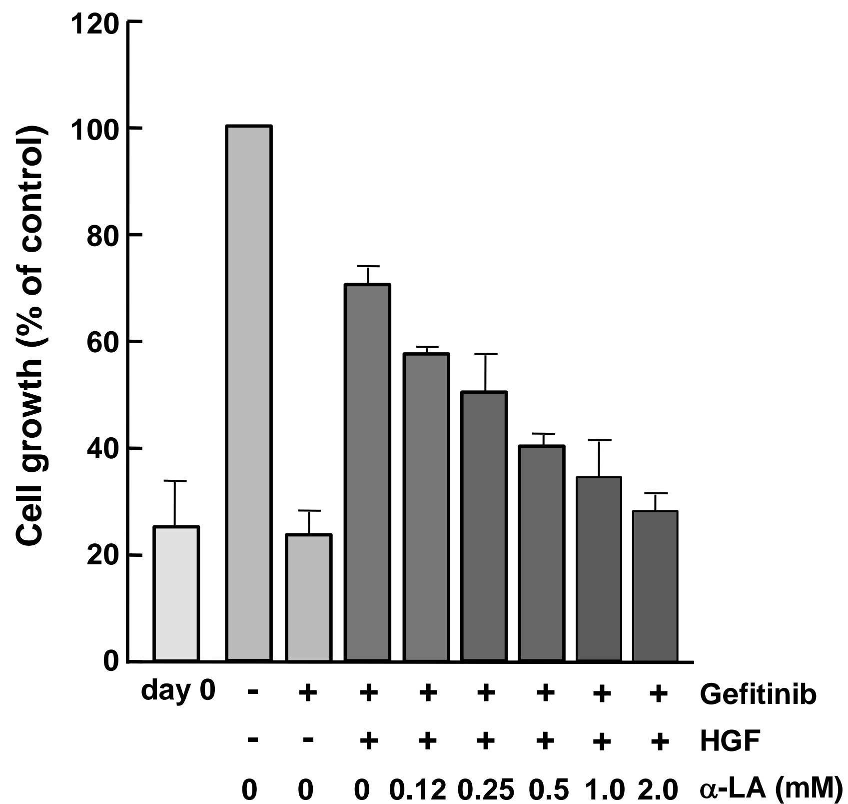


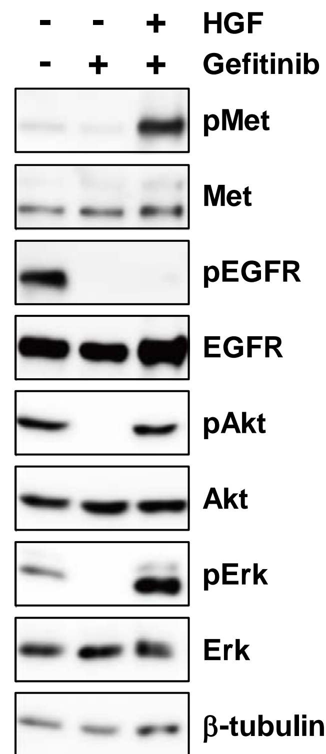


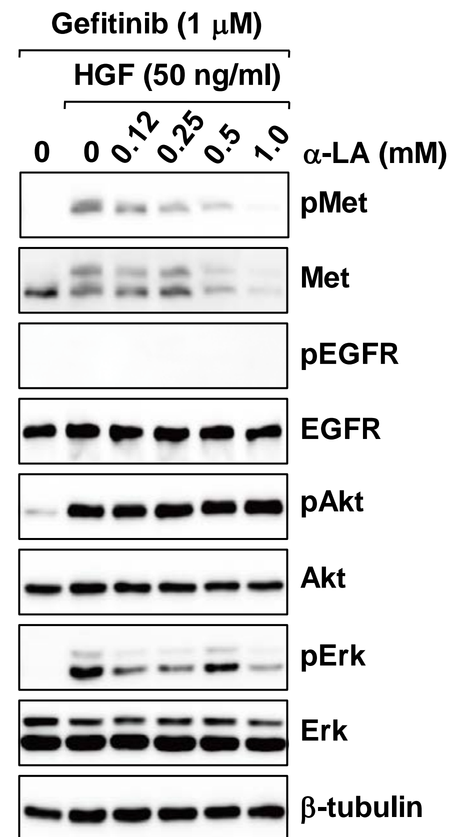
*											*
EGFR	ErbB2	ErbB3	ErbB4	FGFR1	FGFR2a	FGFR3	FGFR4	InsulinR	IGF-I R	Axl	Dtk
Mer	Met	MSPR	PDGFRa	PDGFRb	SCFR	Flt-3	M-CSF-R	C-Ret	ROR1	ROR2	Tie-1
Tie-2	TrkA	TrkB	TrkC	VEGFR1	VEGFR2	VEGFR3	MuSK	EphA1	EphA2	EphA3	EphA4
EphA6	EphA7	EphB1	EphB2	EphB4	EphB6	IgG1	IgG2A	IgG2B	IgG	PBS	
*											*


A
None
α-LA (2.0 mM)
1 12 24 1 12 24 (hr)
pMet
Met
pEGFR
EGFR
pAkt
Akt
pErk
Erk
β-tubulin


B
0.125 0.25 0.5 1.0
α-LA (mM)
pMet
Met
pEGFR
EGFR
pAkt
Akt
pErk
Erk
β-tubulin


A
Cell growth (% of control)
120
100
80
60
40
20
0
day 0
Gefitinib
HGF
α-LA (mM)
0 0 0 0.12 0.25 0.5 1.0 2.0


B
HGF
Gefitinib
pMet
Met
pEGFR
EGFR
pAkt
Akt
pErk
Erk
β-tubulin


C
Gefitinib (1 μM)
HGF (50 ng/ml)
0 0 0.12 0.25 0.5 1.0
α-LA (mM)
pMet
Met
pEGFR
EGFR
pAkt
Akt
pErk
Erk
β-tubulin