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PRECLINICAL STUDIES

Lovastatin overcomes gefitinib resistance in human non-small cell lung cancer cells with *K-Ras* mutations

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Summary Lovastatin is a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor. Its inhibitory action on HMG-CoA reductase leads to depletion of isoprenoids, which inhibits post-translational modification of RAS. In this study, we investigated the effect of combining lovastatin with gefitinib on gefitinib-resistant human non-small cell lung cancer (NSCLC) cell lines with *K-Ras* mutations. Antitumor effects were measured by growth inhibition and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Effects on apoptosis were determined by flow cytometry, DNA fragmentation, and immunoblots. Protein levels of RAS, AKT/pAKT, and RAF/ERK1/2 in cancer cells were analyzed by immunoblot. Compared with gefitinib alone, a combination of gefitinib with lovastatin showed significantly enhanced cell growth inhibition and cytotoxicity in gefitinib-resistant A549 and NCI-H460 human NSCLC cells. In addition, lovastatin combination treatment significantly increased gefitinib-related apoptosis, as determined by fluorescence microscopy and flow cytometric analysis. These effects correlated with up-regulation of cleaved caspase-3, poly (ADP-ribose) polymerase (PARP), and Bax and down-regulation of Bcl-2. The combination of lovastatin and gefitinib effectively down-regulated RAS protein and suppressed the phosphorylation of RAF, ERK1/2, AKT, and EGFR in both cell lines. Taken together, these results suggest lovastatin can overcome gefitinib resistance, in NSCLC cells with *K-Ras* mutations, by down regulation of RAS protein, which leads to inhibition of both RAF/ERK and AKT pathways.

Keywords Lovastatin · Gefitinib · KRAS · Lung cancer

Introduction

Gefitinib, a tyrosine kinase inhibitor (TKI) of the epidermal growth factor receptor (EGFR), has a single agent activity in non-small cell lung cancer (NSCLC) and is currently used for treatment of patients with advanced NSCLC who had previously received platinum-based chemotherapy [1]. Several studies have suggested a high response to gefitinib is most frequently found in persons with adenocarcinoma that have never smoked [2]. Additionally, recent studies showed somatic mutations in exons 18 to 21, close to the region coding the adenosine triphosphate–binding pocket of the kinase domain of EGFR, are associated with response and survival in patients treated with gefitinib [3]. In contrast, the *K-Ras* gene mutation, which occurs in 20% to 30% of NSCLCs and mainly in adenocarcinomas and smokers, has been associated with resistance to gefitinib [4]. Proteins of the RAS super family and subsequent pathways are involved in diverse cellular functions such as cell motility, adhesion, and proliferation [5]. Therefore, activating mutations of the *K-Ras* oncogene are thought to play a role in tumor progression and aggressive behavior.

Lovastatin is an irreversible inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and has been used to treat hypercholesterolemia by blocking the mevalonate biosynthesis pathway [6]. It has also been shown to induce apoptosis in several tumor types [7]. Lovastatin affects the synthesis of other products of the mevalonate pathway such as isoprenoids, which are used as

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substrates for prenylation. Attachment of isoprenoids to RAS proteins facilitates their anchoring to the cell membrane where they carry out their roles [8]. By interrupting the biosynthesis of mevalonate, lovastatin inhibits activation of RAS and downstream signaling cascades, including the RAF/MEK/ERK and PI3K/AKT pathways, which play critical roles in regulation of cell survival and proliferation [7]. Therefore, lovastatin seems to be a promising therapeutic approach for overcoming tumor resistance associated with RAS activation. In fact, a recent *in vitro* study showed that targeting HMG-CoA reductase using lovastatin induces a tumor specific apoptotic response in a variety of tumor cell lines and potentiates the anti-cancer effects of gefitinib [7]. In this study, we evaluated the effects of lovastatin combined with gefitinib in two human NSCLC cell lines harboring *K-Ras* gene mutations and investigated the mechanisms underlying the observed synergic interaction.

Materials and methods

Cell culture

Human NSCLC lines (NCI-H358, Calu-3, NCI-A549, and NCI-H460) were cultured in RPMI medium with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) and maintained at 37°C in a humidified 5% CO_2, 95% air incubator. Gefitinib was provided as a pure powder by AstraZeneca pharmaceuticals (Macclesfield, UK). Lovastatin was purchased from Sigma-Aldrich (St. Louis, MO). The following primary antibodies were used: caspase-3, PARP, phospho-Akt, Akt, p21-RAS, phospho-Raf, phospho-ERK 1/2, ERK 1/2, EGFR (Cell Signaling Technology, Danvers, MA); Bcl-2, Bax, actin (Santa Cruz Biotechnology, Santa Cruz, CA); and phospho-EGFR (BD Pharmingen, Franklin Lake, NJ).

Cell cytotoxicity assay

Cells were seeded (5×10^3 cells per well in 150 μl) in a 96-well flat-bottomed plate (Nunc, Naperville, IL). The cells were incubated overnight to allow for cell attachment and recovery. Following treatment, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium substrate (Sigma) was added and cells were incubated for up to 6 h at 37°C. The resulting formazan precipitate was solubilized by the addition of 100 μl of 0.01 M HCl/10% SDS (Sigma) solution and then analyzed on a MRX Microplate Reader from Dynex Technologies (West Sussex, UK) to determine the absorbance of the samples at 570 nm. The quantity of formazan product was directly proportional to the number of living cells in culture. Drug interaction between gefitinib and lovastatin was assessed using combination index (CI) analysis, where CI <1, CI=1, and CI >1 indicated synergistic, additive, and antagonistic effects, respectively [9].

Cell growth assay

Cells were seeded at 25,000–40,000 per well in a 12-well plate. The next day, the medium was changed to full propagation medium with 10% FBS containing the indicated doses of gefitinib and/or lovastatin, or DMSO as a control. Cells were manually counted on days 1 and 2 with a hemocytometer. Experiments were repeated at least three times and performed each time in triplicate.

Hoechst 33342 staining

Apoptotic cells were assessed using Hoechst 33342 (Sigma-Aldrich) DNA staining. In brief, cells were grown in 6-well plates and stained with Hoechst 33342. A cell that is undergoing apoptosis demonstrates nuclear condensation and DNA fragmentation, which can be detected by fluorescence microscopy. The numbers of apoptotic nuclei in 5 randomly selected fields (objective lens, 2005) were counted, and the number of cells with apoptotic characteristics was expressed as a percentage of the total number of cells examined.

Fluorescence activated cell sorting (FACS) analysis

Cells were trypsinized and suspended in phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, pH 7.4, 145 mM NaCl, and 2.7 mM KCl) containing 2.5 mM ethylene diamine tetra-acetic acid (EDTA), 2.5 mM ethylene glycol tetra-acetic acid (EGTA), and 1% BSA. To determine apoptosis, cells were incubated with 10 μl/ml annexin V-FITC and 6 μl/ml propidium iodide (PI) for 10 min at room temperature, followed by FACS analysis (Coulter, Marseille, France). For FACS analysis of sub-G_1 DNA contents, cells were fixed with 70% ethanol overnight at −20°C and stained with 0.5 ng/ml PI plus 0.5 mg/ml RNase A.

Western blot analysis

After washing with ice-cold PBS, cells were lysed with 2× sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer (20 mM Tris, pH 8.0, 2% SDS, 2 mM dithiothreitol, 1 mM Na_3VO_4, 2 mM EDTA, and 20% glycerol) and boiled for 5 min. The protein concentration of each sample was determined using a Micro-BCA protein assay. In all, 30 μg of total cellular protein was loaded on a 10% SDS-PAGE gel and separated electropho-

retically. Proteins were transferred to polyvinylidene difluoride membranes. Membranes were blocked overnight at 4°C in 20 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween 20 (TBST) containing either 5% BSA (for immunoblotting with anti-phospho-Akt antibody) or 5% nonfat dried milk (for immunoblotting with other antibodies). Membranes were incubated with various antibodies for 1 h at 37°C. Primary antibodies were detected by horseradish peroxidase-conjugated secondary antibodies and, after three washes with TBST, positive signals were visualized using the enhanced chemiluminescence method.

Statistical analysis

All data were expressed as mean±SD obtained from at least three independent experiments. Two-tailed, paired Student's *t* test and all-pairwise, one-way ANOVA test were used to determine the differences between control and treatment groups. All statistical tests were based on two-tailed probability. Additionally, post hoc analyses were performed with Tukey's HSD (Honestly Significant Difference) test to compare the combined treatment group with each of the other treatment conditions.

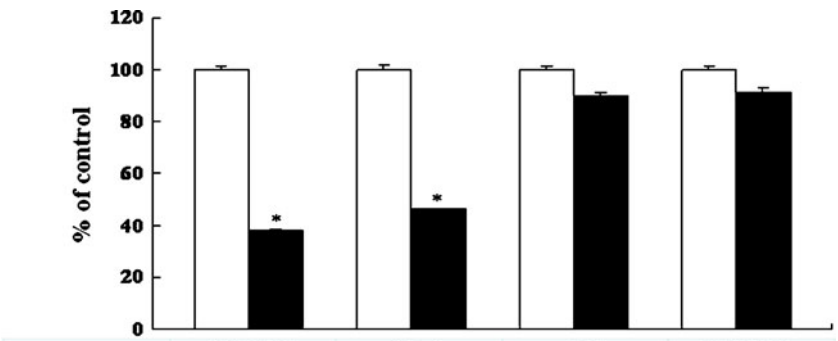
Results

Synergic interaction between gefitinib and lovastatin in gefitinib resistant human NSCLC cell lines

To determine the sensitivity to gefitinib, various human NSCLC cell lines including H358, Calu-3, A549, and H460 cells were exposed to gefitinib (1 μM) for 48 h. As demonstrated in Fig. 1, A549 and H460 cells had an IC_{50}>1 μM, which suggested relatively more resistance to gefitinib, whereas H358 and Calu-3 cells had an IC_{50}<1 μM, which suggested relatively more sensitivity to gefitinib. The gefitinib concentration of 1 μM was used as the cut-off for sensitivity because this concentration approaches levels observed in serum from patients under treatment and has been used in other studies to distinguish between sensitive and resistant cell lines [10]. Both A549 and H460 cell lines are known for harboring *K-Ras* mutations, but containing wild type *EGFR* and *p53* [11]. We next examined the combined effect of lovastatin and gefitinib in these relatively more resistant cell lines. Cells were treated with lovastatin (5 μM in A549, 1 μM in H460), gefitinib (1 μM), or a combination of both drugs for 48 h. As shown in Fig. 2, the combination of gefitinib and lovastatin induced growth inhibition (Fig. 2a) and cytotoxicity (Fig. 2b) in both cell lines that was higher than that induced by gefitinib or lovastatin alone. The CIs determined by the individual responses and the responses to the combination clearly showed synergy with values of 0.13 for A549 and 0.12 for H460 cells, respectively.

Lovastatin enhanced gefitinib-induced apoptosis in gefitinib resistant human NSCLC cells

Flow cytometry analysis was performed to determine whether lovastatin increased gefitinib- induced apoptosis. Cells were incubated for 48 h with a fixed concentration of gefitinib (1 μM) with or without lovastatin (5 μM in A549, 1 μM in H460). As shown in Fig. 3a, the addition of lovastatin significantly increased the percentages of apoptotic cells in both cell lines by 31% (A549) and 8% (H460), respectively compared to gefitinib alone (P<0.001 and P=0.003, respectively). In agreement with these results, the combination of lovastatin and gefitinib enhanced the percentage of cells with nuclear condensation and fragmentation visualized by Hoechst 33342 staining (Fig. 3b). Using immunoblot analysis, we also found lovastatin enhanced the expression of cleaved caspase-3 and cleaved PARP, which represented an active apoptotic



	NCI-H358	Calu-3	A549	NCI-H460
Control (□)	100	100	100	100
Gefitinib 1μM (■)	37.73	46.43	89.62	91.14

Fig. 1 Cytotoxic effects of gefitinib on NSCLC cell lines. Growth assays were performed on NSCLC cell lines including NCI-H358, Calu-3, A549, and NCI-H460 while incubated with gefitinib (1 μM) or DMSO (control). Cells were counted on day 2. Results are expressed as the percentage of living cells compared to the control. Numerical depiction of results is displayed below the graph. *, Significant difference from the control group, P<0.05. *Bars*, SE

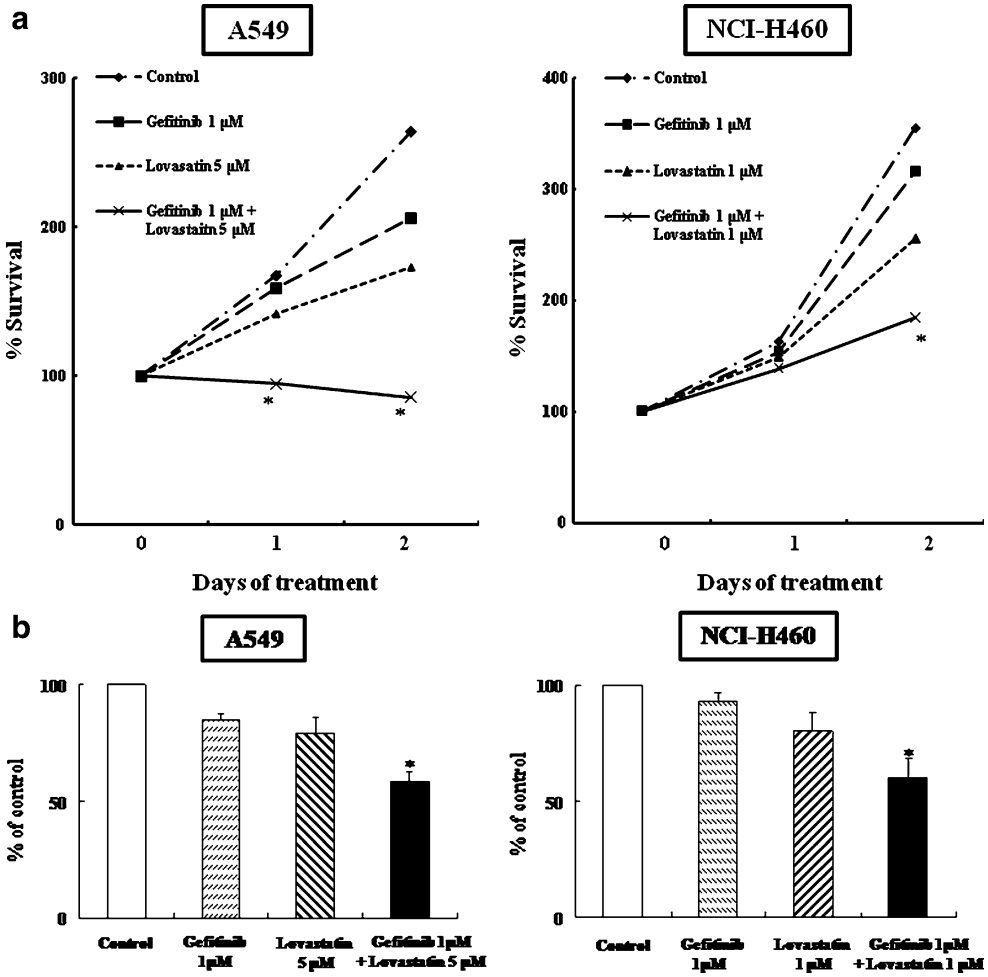


Fig. 2 Cytotoxic effects of gefitinib, lovastatin, and their combination on gefitinib-resistant NSCLC cell lines. **a** Growth assays were performed on A549 and NCI-H460 cells. Cells were seeded in 12-well plates and propagated in full serum (10% FBS) in the presence of the indicated doses of gefitinib, lovastatin, their combination, or DMSO (control) for the indicated time points (24 and 48 h). **b** MTT analysis of gefitinib, lovastatin, and their combination treatment (with 10% FBS) for 48 h. Results are expressed as the percentage of live cells compared to the control. Numerical depiction of results is displayed below the graph. *, Significant difference from the control group, $P<0.05$. *Bars*, SE

process in these cells (Fig. 3c). The anti-apoptotic protein, Bcl-2, plays a major role in evading apoptosis and prolonging survival of cancer cells, in contrast to the pro-apoptotic protein, Bax [12]; therefore, we examined whether lovastatin could modulate the expression of these proteins in gefitinib-resistant lung cancer cells. Immunoblot analysis showed Bcl-2 was substantially down-regulated by lovastatin combination treatment in both cell lines, whereas Bax was up-regulated in both cell lines (Fig. 3c).

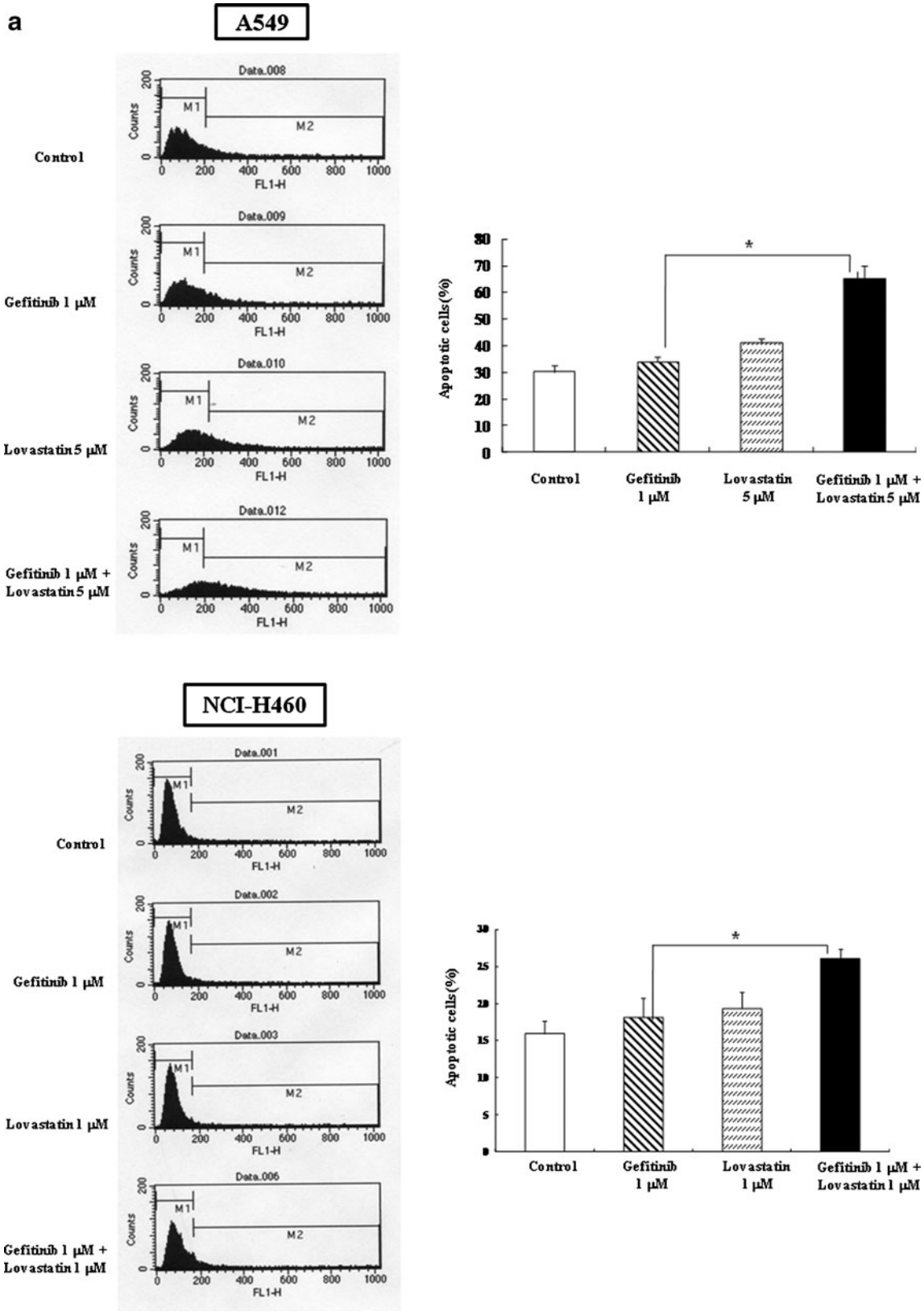
Effects of lovastatin on Ras protein expression and downstream signaling pathways

K-RAS is a downstream mediator of EGFR-induced cell signaling, and *K-Ras* oncogene mutations confer constitutive activation of the signaling pathways without EGFR activation [13, 14]. Growing evidence indicates *K-Ras* mutations are associated with a lack of sensitivity to EGFR-TKIs such as gefitinib or erlotinib [4]. As both A549 and H460 cells harbor activated *K-Ras* oncogene mutations, we examined the effects of lovastatin on RAS protein expression and both MEK/Erk and PI3K/Akt pathways, which are known to be activated by the RAS protein. Cells were incubated with gefitinib, lovastatin, or a combination of both drugs for 48 h. Treatment with lovastatin effectively down-regulated RAS protein expression and inhibited EGF-induced phosphorylation of EGFR, Raf/Erk, and Akt compared with the control or gefitinib alone (Fig. 4).

Discussion

Alternative mechanisms which persistently activate EGFR downstream signaling, including both RAS/Erk and PI3K/

Fig. 3 Effects of gefitinib, lovastatin, and their combination on apoptosis. A549 and NCI-H460 cells were treated with gefitinib, lovastatin, and their combination for 48 h. **a** Cells were trypsinized, loaded with propidium iodide and annexin V, and then analyzed by flow cytometry. The number of living cells and early apoptotic cells are expressed as a percentage of the total cell number. *, $P<0.05$, paired *t* test. *Bars*, SE. **b** Morphological change of cells detected by Hoechst 33258 staining. All fields are representative of multiple fields observed in three independent experiments. **c** Cell lysates were analyzed by Western blotting with the indicated antibodies


a
A549
Control
Gefitinib 1 μM
Lovastatin 5 μM
Gefitinib 1 μM + Lovastatin 5 μM
Data.008
Data.009
Data.010
Data.012
M1
M2
Counts
FL1-H
Apoptotic cells(%)
Control
Gefitinib 1 μM
Lovastatin 5 μM
Gefitinib 1 μM + Lovastatin 5 μM
NCI-H460
Control
Gefitinib 1 μM
Lovastatin 1 μM
Gefitinib 1 μM + Lovastatin 1 μM
Data.001
Data.002
Data.003
Data.006
Apoptotic cells(%)
Control
Gefitinib 1 μM
Lovastatin 1 μM
Gefitinib 1 μM + Lovastatin 1 μM

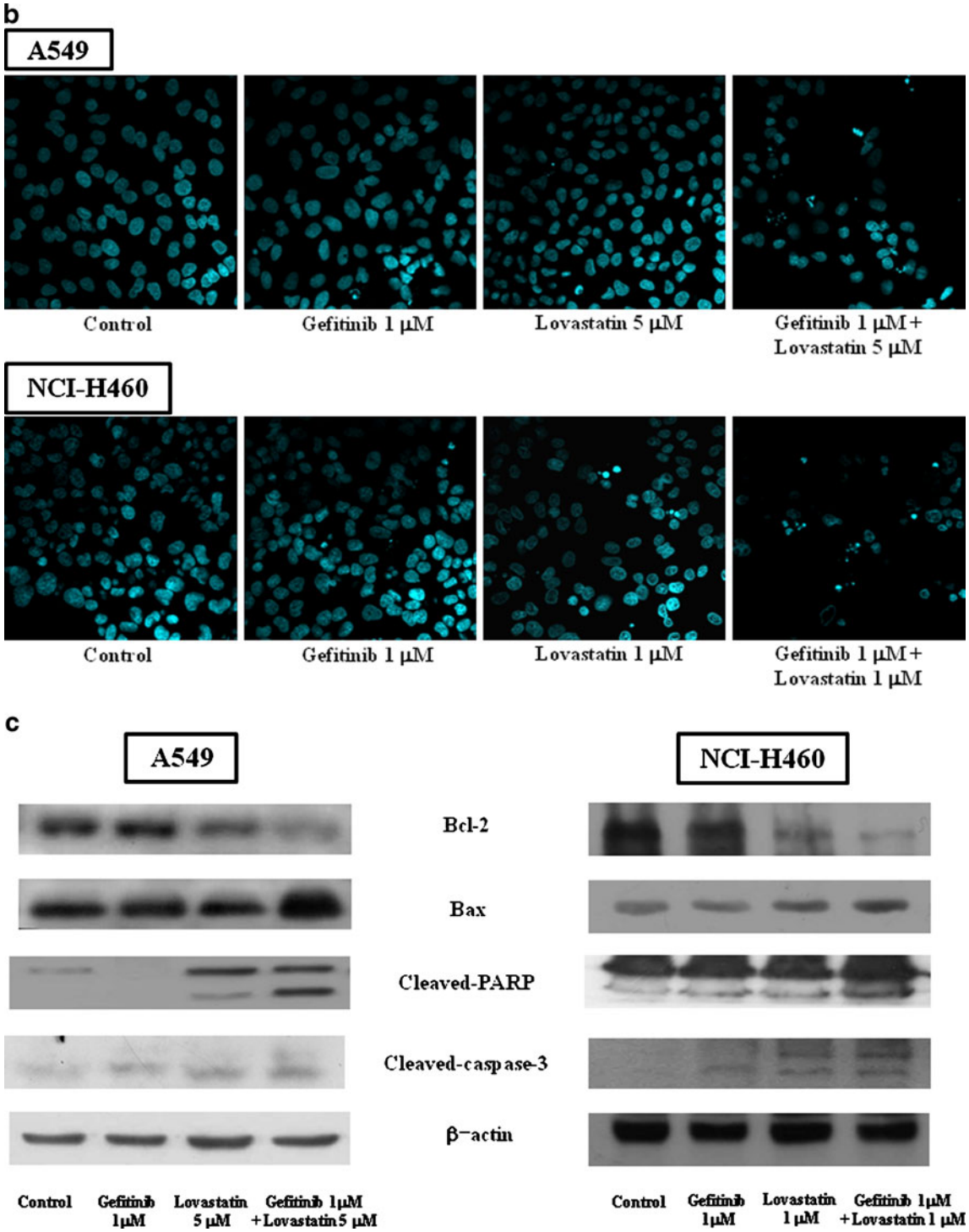


Fig. 3 (continued)

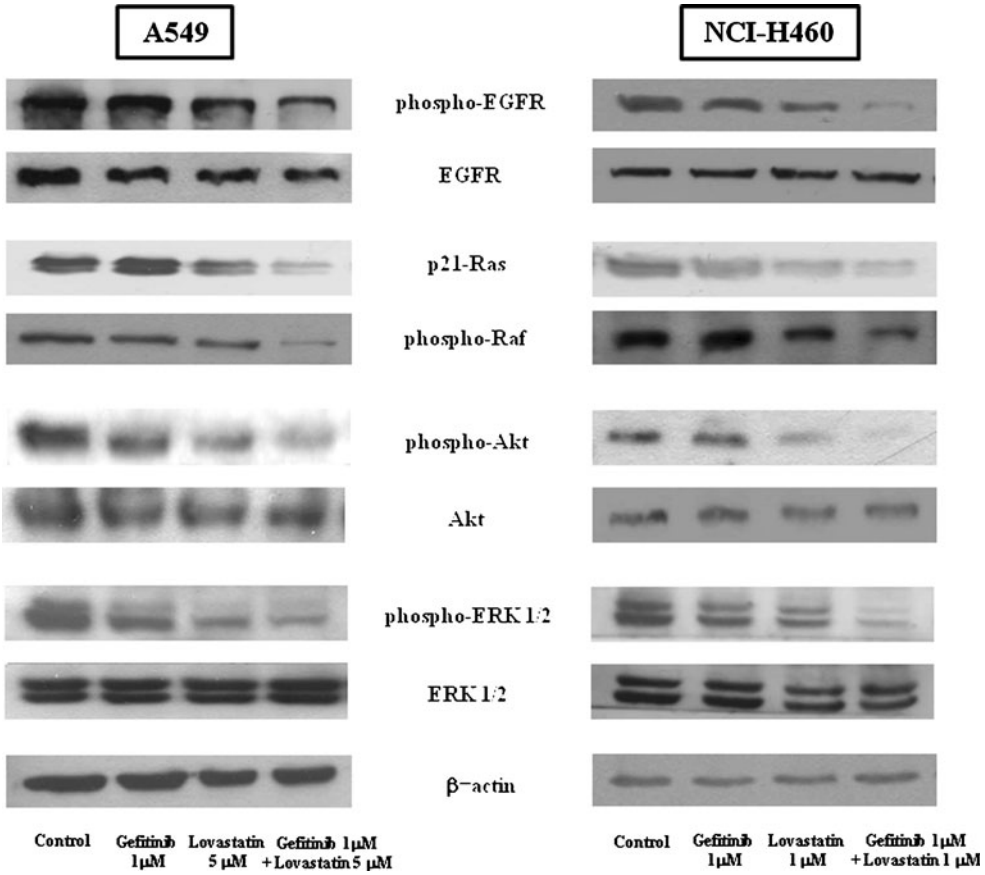


Fig. 4 Effects of gefitinib, lovastatin, and their combination on RAS protein and EGFR-signaling pathways. A549 and NCI-H460 cells were treated with gefitinib, lovastatin, or their combination for 48 h. The change in RAS and EGFR-related signals was analyzed by Western blotting

Akt kinase pathways, are the main reasons for gefitinib resistance [4, 13]. Therefore, simultaneous inhibition of both pathways would more effectively reduce tumor cell survival. The Ras/MAPK/Erk and PI3K/Akt pathways are major signaling networks linking EGFR activation to cell proliferation and survival [15] and both pathways are regulated by the small GTPase, RAS [16]. Previous studies have revealed *K-Ras* gene mutations cause constitutive activation of the protein products, resulting in excessive activation of its downstream pathways which promotes cancer development [14, 17]. In addition, *EGFR* and *K-Ras* mutations are rarely found in the same tumor, suggesting they have functionally equivalent roles in lung tumorigenesis [18]. According to prior clinical studies with EGFR-TKIs in NSCLC, harboring *K-Ras* mutations was associated with worse clinical outcomes when treated with gefitinib or erlotinib therapy [4, 18, 19]. Furthermore, a recent systemic review and meta-analysis of studies in advanced NSCLC and metastatic colorectal cancer provided empirical evidence that *K-Ras* mutations are highly specific negative predictors for response to EGFR targeted therapies, including both TKIs and antibodies [20].

RAS proteins require several post-translational modifications such as protein prenylation for membrane binding and full biological activity, and these processes are catalyzed by farnesyltransferase (FTase) and geranylgeranyltransferase I (GGTase I) [21]. Lovastatin, an irreversible competitive inhibitor of HMG-CoA reductase, blocks the conversion of HMG-CoA to mevalonate and, thus, synthesis of downstream mevalonate products such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate which are the main substrates of FTase and GGTase I. By eliminating the intracellular bioavailability of these isoprenoids, lovastatin more effectively inhibits prenylation of signaling molecules, especially RAS and RAS related proteins [8]. In general, the anti-tumor effects of statins, including lovastatin, are thought to be due to inhibition of protein prenylation.

Previously Mantha et al. demonstrated the combination of gefitinib and lovastatin showed significant synergic cytotoxic effects *in vitro* in a total of 16 squamous cell carcinomas, NSCLC, and colon carcinoma cell lines [7]. While these cell lines did not possess the ATP-binding site activating mutations, which confer increased sensitivity to gefitinib, combining lovastatin with gefitinib induced more significant inhibition of AKT activation than either agent alone [7]. Additionally, lovastatin significantly enhanced the sensitivity to gefitinib treatment regardless of PTEN loss in glioblastoma cell lines [22]. These results suggest

lovastatin can augment EGFR inhibition. However, the exact mechanism for this synergy has not been elucidated.

Prior *in vitro* studies have shown gefitinib effectively blocks Akt and Erk phosphorylation in gefitinib-sensitive NSCLC cell lines. In contrast, persistent activation of the PI3K/Akt and/or Ras/Erk pathways is associated with gefitinib-resistance in NSCLC cell lines. Simultaneous inhibition of both PI3K/Akt and MEK/Erk pathways in gefitinib-resistant NSCLC cell lines more effectively reduces cancer cell survival with gefitinib treatment than inhibition of either pathway alone [11]. In the present study, we used A549 and NCI-H460 cell lines which showed resistance to gefitinib treatment. Both cell lines are characterized by *K-Ras* exon 1 and 2 mutations, but wild type *EGFR*. We found the combination of gefitinib and lovastatin effectively inhibited both RAF/ERK1/2 and PI3K/AKT pathways in A549 and H460 cell lines in our study. In addition, lovastatin combined with gefitinib down-regulated the expression of RAS protein and inhibited the activation of EGFR, an effect not shown in cells treated with gefitinib alone. Upon combination treatment with lovastatin and gefitinib, the loss of RAF/ERK and AKT activation in cells with *K-Ras* mutations resulted in a marked increase in cell proliferation inhibition and apoptosis. Recently, Laezza et al. demonstrated the anti-tumor activity of lovastatin in *K-RAS* dependent thyroid tumors. They found lovastatin blocked RAS activation through inhibition of farnesylation [23]. Ogunwobi et al. reported statins inhibited proliferation and induced apoptosis in esophageal adenocarcinoma cells via inhibition of RAS farnesylation and inhibition of ERK and AKT signaling pathways [24]. These reports further support our finding of synergic cytotoxic activity of lovastatin combined with gefitinib in *de-novo* gefitinib-resistant NSCLC cells harboring *K-Ras* mutations.

In previous phase II studies, HMG-CoA reductase inhibitors alone, such as lovastatin or pravastatin, showed a limited tumor response [25, 26]; however, when combined with various chemotherapeutic agents, they yielded additive or synergistic anti-tumor activity in preclinical models [26]. In addition, a randomized trial of 91 patients with hepatocellular carcinoma revealed that the addition of pravastatin to standard chemotherapy prolonged survival significantly (18 months in the pravastatin group compared with 9 months in the control group, $P=0.006$) [27]. Therefore, it could be an attractive therapeutic approach to combine lovastatin with gefitinib in patients with NSCLC and even in patients with *K-Ras* mutations.

In conclusion, our results suggest the combination of lovastatin and gefitinib are a promising therapeutic strategy to overcome gefitinib resistance and to augment the cytotoxic effects of gefitinib. However, further studies are needed to evaluate and establish the potential clinical benefits of using lovastatin in NSCLC treatment.

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