Introduction

Lung cancer is the most common cancer and the leading cause of cancer-related mortality worldwide. The majority of lung cancers comprise NSCLC (non-small-cell lung cancer), and one-third of these patients are diagnosed with stage III disease when surgical excision is not an option and curative treatment is extremely limited. For these patients, the combined treatment of radiotherapy and chemotherapy are extensively used. Despite the tremendous efforts and progress in lung cancer research and the use of aggressive multimodal chemotherapies and radiotherapies, the overall treatment outcome for these NSCLC patients remains disappointing, with a 5-year survival rate of approximately 15%. However, the discovery of activating epidermal growth factor receptor (EGFR) mutations in NSCLC has led to a paradigm shift in cancer treatment for NSCLC patients. The EGFR family of receptor tyrosine kinases (TKs) consists of four members (EGFR (HER1/ErbB1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4)) that

Original Article

Capilliposide from Lysimachia capillipes inhibits AKT activation and restores gefitinib sensitivity in human non-small cell lung cancer cells with acquired gefitinib resistance

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Abstract

Most gefitinib-treated patients with non-small cell lung cancer (NSCLC) would eventually develop resistance. Lysimachia capillipes (LC) capilliposide extracts from LC Hemsl. show both in vitro and in vivo anti-cancer effects. In this study we investigated whether LC capilliposide in combination with gefitinib could overcome the resistance of NSCLC cells to gefitinib and identified the signaling pathways involved. Treatment with LC capilliposide alone inhibited the growth of a panel of NSCLC cell lines (PC-9, H460, H1975, H1299 and PC-9-GR) sensitive or resistant to gefitinib with IC50 values in the range of μg/mL. In the gefitinib-resistant PC-9-GR cells (which have a T790M EGFR mutation), LC capilliposide (at the IC50, i.e. 1.2 μg/mL) markedly enhanced the inhibitory effects of gefitinib with its IC50 value being decreased from 6.80±1.00 to 0.77±0.12 μmol/L. By using the median effect analysis we showed that combination treatment of LC capilliposide and gefitinib could restore gefitinib sensitivity in PC-9-GR cells. Furthermore, LC capilliposide (1.2 μg/mL) significantly increased the apoptotic responses to gefitinib (0.77 μmol/L) in PC-9-GR cells, but did not affect gefitinib-induced G0/G1 arrest. Moreover, LC capilliposide (1.2 μg/mL) in combination with gefitinib (0.77, 1.0 μmol/L) markedly decreased the phosphorylation of the EGFR downstream signaling molecule AKT, which neither LC capilliposide nor gefitinib alone affected. In PC-9-GR cells with siRNA knockdown of AKT, addition of LC capilliposide was unable to increase gefitinib sensitivity. In a PC-9-GR xenograft mouse model, combination treatment with LC capilliposide (15 mg·kg-1·d-1, ip) and gefitinib (50 mg·kg-1·d-1, ip) dramatically enhanced tumor growth suppression (with a TGI of 109.3%), compared with TGIs of 22.6% and 56.6%, respectively, in mice were treated with LC capilliposide or gefitinib alone. LC capilliposide can restore the cells’ sensitivity to gefitinib through modulation of pAKT levels, suggesting that a combination of LC capilliposide and gefitinib may be a promising therapeutic strategy to overcome gefitinib resistance in NSCLCs with a T790M mutation.

Keywords: Lysimachia capillipes; capilliposide; non-small cell lung cancer; gefitinib resistance; T790M; AKT; PC-9-GR xenograft mouse model

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regulate many developmental, metabolic and physiological processes. The intracellular TK activity of EGFR is increased as a consequence of the binding of various cognate ligands, which include EGF, transforming growth factor-α, amphiregulin and others, leading to either the homodimerization of two EGFRs or the heterodimerization of EGFR with other family members[6]. The activation of receptor TKs results in the autophosphorylation of the intracellular domain of EGFR, and the resulting phosphoryrosine residues act as a docking site for various adapter molecules, leading to the activation of the Ras/mitogen-activated protein kinase pathway, the PI3K/AKT pathway and signal transducers and activators of transcription signaling pathways[7-9]. Mutations in the region of the EGFR gene that encode the TK domain of the receptor alter the enzymatic TK pocket of the receptor, resulting in constitutive activation; furthermore, these mutations are found in approximately 10% of NSCLCs in Caucasians and 30% of NSCLCs in East Asians[10]. A number of retrospective reviews and prospective trials have established that treatment with gefitinib or erlotinib (first-generation reversible EGFR TKIs) leads to radiographic responses in 75%-80% of patients with NSCLCs with EGFR mutations[11-16]. However, the initial response to first-generation TKIs is often limited with duration of 10-16 months due to acquired resistance, and almost 50% of cases are caused by an acquired or de novo T790M mutation[17-20]. Second-generation EGFR TKIs, including the drug afatinib, showed promising results in overcoming T790M drug resistance in preclinical studies and in clinical trials[21-24]. However, the nonspecific reactivity and potential for off-target activity that may cause tissue injury and drug-related toxicities were major concerns for the second-generation covalent TKI drugs[25, 26]

The third-generation EGFR-TKIs, which include AZD9291, CO-1886 and HM61713, were specifically designed to inhibit both activating/sensitizing mutations (EGFRm) and the resistant mutation T790M[27]. AZD9291 has been recently approved by the FDA with an objective response rate of 59% and a response duration of 12.4 months, which provides important new option for patients positive for the T790M mutation[28]. However, the high cost of the drug and its limited availability in a handful of countries is currently the great hurdle in clinical practice. Thus, exploring effective and feasible treatment strategies with few side effects to overcome the resistance to first generation EGFR-TKIs is still of significance for improving the prognosis of patients with NSCLC.

Traditional Chinese medicine (TCM) has a long history of being widely used for treating human diseases, including cancer. Lysimachia capillipes Hemsl grows in southeastern China and has been used extensively as a traditional medicine for treating cough, menstrual symptoms, rheumatalgia disorder and carcinomas. Recently, LC capilliposide extracted from Lysimachia capillipes Hemsl has been tested for its anti-cancer properties[29, 30], and the results revealed both in vitro and in vivo anti-cancer effects of LC capilliposide in prostate, gastric and breast cancer cells[31–33]. Our preclinical study has also demonstrated the potential therapeutic effects of LC capilliposide on human lung cancer cells[34]. In this study, we examined the combined effect of LC capilliposide and gefitinib in NSCLC cells, and our results showed that LC capilliposide not only synergistically enhances the killing effect of gefitinib on NSCLC cells but also restores gefitinib sensitivity to NSCLC cells with acquired gefitinib resistance.

Materials and methods

Cell culture and reagents

The human NSCLC cell lines PC-9, H460, H1975, and H1299 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The human NSCLC cell line PC-9-GR was developed by chronic exposure to gefitinib as we previously reported[35]. All of the cell lines were maintained in RPMI-1640 (Gibco, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Waltham, Massachusetts, USA). Gefitinib (Cayman, Ann Arbor, MI, USA) was dissolved in dimethyl sulfoxide (DMSO). LC capilliposide was obtained from the Department of Chinese Medicine Sciences & Engineering at Zhejiang University (Hangzhou, Zhejiang, China). All of the drugs were diluted with fresh media before each experiment.

Cell growth inhibition assay

Cell proliferation analysis was performed using the MTS assay (tetrazolium-based CellTiter 96 Aqueous One Solution Proliferation assay), as per the manufacturer’s instructions (Promega, Fitchburg, WI, USA). Briefly, cells were plated in a 96-well plate (3000 cells/well). Approximately 24 h after plating, cells were treated with various concentrations of gefitinib and LC capilliposide, and cell viability was determined 72 h later. The IC50 value (defined as the concentration necessary for a 50% reduction in the absorbance) was calculated based on the nonlinear regression fit method by GraphPad Prism 5.0 software (San Diego, CA, USA). For the combination treatment, cells were seeded in a 96-well plate at the density of 3×10³ per well and incubated for 24 h, after which LC capilliposide and gefitinib were added concurrently to the medium and incubated for 72 h before measurement. The half-maximal inhibitory concentration (IC50) was determined with the corresponding dose response data for each cell line.

Median effect analysis

Median effect analysis was performed as described previously[36]. Briefly, cells were treated with increasing total doses of gefitinib and LC capilliposide with a constant dose ratio based on the corresponding IC50 values, and cell viability was determined by the cell proliferation assay (tetrazolium-based CellTiter 96 Aqueous One Solution Proliferation assay, Promega, Fitchburg, WI, USA). A plot of the log of the total dose versus the log of the reciprocal of the cell fraction affected minus 1 yielded a linear plot. The slope and y-intercept from these plots were used to calculate the CI (combination index) by using CalcuSyn Version 2.0 software (BioSoft, Great Shalford, Cambridge, UK). The CI values were interpreted as follows: <1.0=synergism; 1.0=additive; >1.0=antagonism.
siRNA transfection
Cells were seeded at a density of 3×10^5 cells/well in 6-well plates. The cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. siRNA-control and siRNA-AKT (Santa Cruz Biotech, Dallas, TX, CA) were used. Western blot analysis was used to verify the gene silencing efficiency. After 48 h, cells were pretreated with gefitinib and LC for cell growth inhibition analysis.

Detection of cell apoptosis
Cells were treated with DMSO, LC capilliposide only (1.20 μg/mL, IC_{50}), gefitinib only (0.77 μmol/L, IC_{50}), or gefitinib (0.77 μmol/L) combined with LC (1.20 μg/mL). Cells were then collected 48 h later and stained with annexin V-FITC (fluorescein isothiocyanate) as per the manufacturer’s instructions (Annexin V-FITC Apoptosis Detection kit; BD Biosciences, USA). Flow cytometric analysis with a Becton Dickinson FACScan cytofluorimeter (BD Biosciences, Franklin Lakes, NJ, USA) was used to determine the percentage of apoptotic cells. Up to 5×10^4 cells were counted for each sample, and the quantification of apoptotic cells was calculated by CellQuest software. Both early apoptotic (annexin V-positive and PI-negative) and late apoptotic (annexin V-positive and PI-positive) cells were included as part of the total apoptosis.

Cell cycle analysis
PC-9-GR cells were seeded into 6-well culture plates overnight, and cells were then treated with 1.20 μg/mL of LC capilliposide, 0.77 μmol/L of gefitinib, or a combination of gefitinib (0.77 μmol/L) and LC capilliposide (1.20 μg/mL) for 24 h. DMSO was included as a control. After the treatments, cells were collected and fixed with 70% ethanol, stained with propidium iodide and analyzed by flow cytometry with 5×10^4 events counted per run. The percentage of cells in the G_s, S, and G_M phases of the cell cycle were determined by using FlowJo software (FlowJo, Ashland, OR, USA).

Western blot analysis
Cell lysates were prepared in RIPA buffer with mild sonication and subjected to SDS-PAGE gel for immunoblot assays. Antibodies against phospho-EGFR, EGFR, phospho-AKT, AKT, phospho-ERK, ERK, and GAPDH were purchased from Cell Signaling Technology (CST, Danvers, MA, USA). Densitometry using Image Lab 5.0 software (BioRad Laboratories, Hercules, CA, USA) was conducted to determine the intensity of the Western blot signal, and the intensity of the target protein was normalized to the corresponding GAPDH band.

Phospho-receptor tyrosine kinase array assay
The Human Phospho-Kinase Array Kit (ARY003B, R&D Systems, Minneapolis, MN, USA) was used to detect the relative levels of phosphorylation for 43 kinase targets as per the manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA). Briefly, cell lysates were diluted to 500 μg of protein per array set and incubated overnight with the array. The array was washed to remove unbound proteins and then incubated with a cocktail of biotinylated detection antibodies. Streptavidin-HRP and chemiluminescent detection reagents were applied, and the signal was captured corresponding to the amount of bound phosphorylated protein using Image Lab 5.0 software (BioRad Laboratories, Hercules, CA, USA).

Tumor growth assay
PC-9-GR cells (1×10^6 in 0.2 mL 1×HBSS+1% HSA) were inoculated subcutaneously into the right thigh of 4- to 6-week-old female Nu/Nu mice (Charles River, Beijing, China). When the average tumor volume reached 200 mm^3, the mice were randomized into 4 groups to receive the following treatments: (a) methylcellullose/Tween 80 as vehicle for 10 days; (b) gefitinib (50 mg·kg^{-1}·d^{-1}) for 10 days; (c) LC (15 mg·kg^{-1}·d^{-1}) for 10 days; and (d) gefitinib (50 mg·kg^{-1}·d^{-1})+LC (15 mg·kg^{-1}·d^{-1}) for 10 days. All of the chemicals were administered by ip injection. Tumors were measured twice weekly, and the tumor volumes were determined from caliper measurements of the tumor length (L) and width (W) according to the formula \( V = \frac{L \times W^2}{2} \). The equation \( \%\text{TGI} = (1 - \text{change of tumor volume in treatment group/change of tumor volume in control group}) \times 100 \) was used to determine the percentage of tumor growth inhibition and antitumor efficacy.

Immunohistochemistry analysis
PC-9-GR xenograft tumors were collected after 10 days of the aforementioned daily treatments and fixed in 4% formalin. Antigen retrieval was conducted on FFPE tissues sections for 5 min with retrieval buffer (DAKO, Glostrup, Denmark) followed by washing under running water for 5 min. Tissue samples were then rinsed in PBS containing 1% Tween (TBST) and incubated with endogenous peroxidase blocker on a LabVision autostainer for 10 min. Slides were washed twice in TBST, incubated with primary antibodies against pEGFR, pAKT, or CC3 (Cell Signaling Technology) for 60 min at room temperature and then washed twice in TBST. DAKO EnVision™+System-HRP was used as the secondary antibody for visualization, and staining was detected using diaminobenzidine (Dako). For Ki67 immunohistochemical analysis, tumor sections were incubated with biotinylated primary antibody (Dako) for 1 min at room temperature and then washed twice with TBST. Following a 15-min streptavidin-peroxidase treatment and washing with TBST, the slides were counterstained with DAPI and visualized by chemiluminescence. For analysis of the baseline expression or modulation, IHC scoring of phospho-EGFR and phospho-AKT was conducted using the following formula: scoring=0×[% cells with no staining (0)]+1×[% cells staining faint to barely visible (1)]+2×[% cells staining weak to moderately (2)]+3×[% cells staining strongly (3+)]. The samples were analyzed by 2 separate pathologists using microscopy. Quantification of Ki67- and CC3-positive signals was conducted using the ARIOL system (Genetix, San Jose, CA, USA).
Statistical analysis

Data were presented as the means±SD from at least three independent experiments. Student’s t-test was used to determine the significance between groups. *P*<0.05 was defined as statistically significant.

Results

Effects of LC capilliposide and gefitinib on growth of NSCLC cells

We first determined the inhibitory effects of gefitinib and LC capilliposide on the growth of a panel of five NSCLC cell lines. Of these cell lines, H1299 expresses wild-type EGFR, PC-9 expresses mutant EGFR with a 15-bp deletion (EGFR-19Del), H1975 and PC-9-GR express mutant EGFRs with dual mutations, one of which includes T790M (EGFR-L858R/T790M for H1975 and EGFR-19Del/T790M for PC-9-GR)\(^3\), and H460 expresses mutant KRAS (KRAS-Q61H). With IC\(_{50}\) values determined by the MTS assay, we found that these cells responded to gefitinib treatment with a wide range of IC\(_{50}\) values, but only PC-9 cells showed an IC\(_{50}\) value at the nmol/L level (31.00±6.15 nmol/L). Of note, the MTS assay showed that the IC\(_{50}\) values of the other four cell lines ranged from near 7 to 16 μmol/L, indicating that these cells are relatively resistant to gefitinib treatment (Figure 1A and Table 1). However, no significant difference was observed for the IC\(_{50}\) values of LC capilliposide determined in these tested cells, as the IC\(_{50}\) values for LC capilliposide in all five cell lines were in the range of μg/mL (Figure 1B and Table 1).

| Cell lines | EGFR | KRAS | Gefitinib IC\(_{50}\) (μmol/L) | LC capilliposide IC\(_{50}\) (μg/mL) | LC capilliposide IC\(_{30}\) (μg/mL) |
|------------|------|------|-------------------------------|---------------------------------|---------------------------------|
| H1299      | Wild type | Wild type | 12.75 | 6.83 | 3.92 |
| H460       | Wild type | Q61H | 15.63 | 5.65 | 3.22 |
| H1975      | L858R/T790M | Wild type | 12.01 | 3.51 | 2.00 |
| PC-9-GR    | 19Del/T790M | Wild type | 6.80 | 2.10 | 1.20 |
| PC-9       | 19Del | Wild type | 0.031 | 1.90 | 1.09 |

Figure 1.

Cytotoxic effects of gefitinib and LC capilliposide on NSCLC cells and the synergy of LC capilliposide and gefitinib to sensitize NSCLC cells to gefitinib treatment. Cells were cultured in 96-well plates and were treated with different doses of gefitinib (A) or LC capilliposide (B) for 72 h. (C) Effects of LC capilliposide on cell growth inhibition in NSCLC cells treated with different doses of gefitinib. Cells were treated with a combination of LC capilliposide (at doses of IC\(_{50}\)) and different concentrations of gefitinib for 72 h. Error bars indicate the standard deviation of three independent measurements. (D) Median effect analysis. The combination index (CI) was determined in PC-9-GR cells treated with LC capilliposide and gefitinib. (E) The CI was determined in PC-9 cells treated with LC capilliposide and gefitinib.

Combination treatment of LC capilliposide and gefitinib inhibits cell growth of NSCLC cells with acquired gefitinib-resistance

We next examined the potential effects of LC capilliposide on the growth of four gefitinib-resistant cell lines and one sensitive cell line in response to gefitinib treatment. In this study, we used the IC\(_{30}\) values as the appropriate dose for LC capilliposide treatment, and we found no obvious inhibitions on cell growth for all of the tested cell lines when they were treated with LC capilliposide alone for 72 h. However, we noticed that the presence of LC capilliposide increased the inhibitory effects of gefitinib in these cells, with decreases of the IC\(_{50}\) values from 6.80±1.00 to 0.77±0.12 μmol/L for PC-9-GR, 12.01±2.80 to 2.80±0.30 μmol/L for H1975, 12.75±2.10 to 6.0±0.87 μmol/L for H1299, 15.63±2.53 to 12.5±1.02 μmol/L.
LC capilliposide enhances the apoptotic response to gefitinib treatment in PC-9-GR cells

To investigate the potential mechanisms of the enhancing effect of LC capilliposide on the cytotoxicity of gefitinib in PC-9-GR cells, we performed an apoptosis analysis in cells after treatment with 1.2 μg/mL LC capilliposide alone, 0.77 μmol/L gefitinib alone (ie, the IC_{50} value in the presence of LC capilliposide), or a combination of both drugs. As shown in Figure 2A, we detected a significantly higher percentage (34.6±2.0) of total apoptosis in cells treated with the combination of gefitinib and LC capilliposide for 24 h when compared to that in cells treated with either gefitinib (14.7±1.4) or LC capilliposide alone (14.3±1.3). The combination treatment with gefitinib and LC capilliposide also induced a higher percentage (26.6±1.7) of early apoptosis (versus 8.7±1.0 for gefitinib alone and 7.5±1.2 for LC capilliposide alone) (Figure 2B). These results indicate that LC capilliposide enhances the apoptotic response to gefitinib treatment in PC-9-GR cells.

However, no obvious effect of LC capilliposide was observed on gefitinib-induced G_{0}/G_{1} arrest in PC-9-GR cells (Figure 2C).

LC capilliposide modulates gefitinib-inhibited EGFR downstream signaling pathways in PC-9-GR cells

We next determined the effects of the combination treatment (gefitinib+LC capilliposide) on activation of the EGFR downstream signaling pathways in PC-9-GR cells. In this experiment, we also used 1 μmol/L of gefitinib, which is the maximum clinical dose, as a treatment option. Our results showed that treatment with gefitinib alone, at both doses of 0.77 and 1 μmol/L, reduced the phosphorylation of EGFR, AKT and ERK1/2 in PC-9-GR cells. The presence of LC capilliposide alone did not elicit any changes for the gefitinib-affected phosphorylation of ERK1/2; however, LC capilliposide further down-regulated the phosphorylation level of AKT in cells when combined with gefitinib (P<0.05, Figure 3A and 3B). The phospho-kinase array (Figure 3C and Supplementary Figure S1) also showed a significant decrease of AKT phosphorylation in PC-9-GR cells treated with the combination of gefitinib and LC capilliposide when compared to that of gefitinib treatment alone. In addition, the array results further revealed that the LC capilliposide treatment could lead to enhanced inhibitory effects of gefitinib on the phosphorylation of WNK1 and PRAS40.

To further evaluate the role of pAKT on the restoration of LC-induced sensitivity to gefitinib in PC-9-GR cells, we decreased the phosphorylation of AKT by knocking down AKT with siRNA transfection. Compared with the PC-9-GR cells with siRNA knockdown of AKT and treated with gefitinib, the addition of LC was unable to increase the sensitivity to gefitinib (Figure 4). Thus, our results indicate that LC exposure can restore the cells’ sensitivity to gefitinib through modulation of pAKT levels.

Effects of LC capilliposide on gefitinib-inhibited tumor growth of PC-9-GR xenografts in vivo

Studies were extended to an in vivo xenograft mouse model. We investigated the potential effect of LC capilliposide on tumor growth of PC-9-GR xenografts in response to gefitinib treatment. Our results showed that treatment with either LC capilliposide (15 mg/kg for 10 d) or gefitinib (50 mg/kg for 10 d) alone could inhibit in vivo PC-9-GR tumor growth with TGI of 22.6% and 56.6%, respectively. The combination treatment, however, dramatically increased tumor growth suppression (with a TGI of 109.3%) when compared to the treatment with gefitinib or LC capilliposide alone (P<0.05) (Figure 5A). In this experiment, we also measured the mouse body weight to assess the tolerability of systemic therapies, and no obvious body weight changes were observed (Supplementary Figure S2), suggesting that cotreatment with gefitinib and LC capilliposide is well tolerated.

We also performed immunohistochemistry (IHC) to detect the phosphorylation of EGFR and AKT, the cell proliferation marker Ki67 and the apoptotic marker CC3 (cleavage of caspase 3) in tumor specimens that were collected from PC-9-GR xenograft tumors with the indicated treatments. Our results showed that treatment with gefitinib alone suppressed phosphorylation levels of EGFR and AKT. Gefitinib treatment also decreased Ki67 expression in the PC-9-GR xenograft tumors. Treatment with LC capilliposide alone resulted in a significantly reduced phosphorylation level of AKT and also caused slightly decreases of EGFR phosphorylation and Ki67 expression. Of note, the combination treatment further decreased AKT phosphorylation and Ki67 expression; however, it did not cause an obvious change in EGFR phosphorylation when compared to each single treatment in the PC-9-GR xenograft tumors. In addition, we observed an increase of positive CC3 staining in the tumor specimens of the xenograft subjected to the combination treatment (Figure 5B and Supplemental Table 1).

Taken together, our data suggest that LC capilliposide can
enhance gefitinib-inhibited AKT signaling and restore the gefitinib sensitivity to NSCLC cells with acquired gefitinib resistance.

**Discussion**

The empirical criteria for defining clinical subtypes of lung cancer are gradually transiting from histopathology to genetic variations in driver genes. Targeting these driver mutations, such as sensitizing EGFR mutations, has dramatically improved the prognosis of patients with advanced NSCLC. To date, clinical trials have confirmed that EGFR-TKI therapy with first-generation TKIs is superior to chemotherapy as a first-line treatment, with an increase in progression-free survival (PFS) and objective response rate (ORR) of approximately 25% in NSCLC patients with EGFR mutations\(^{37,38}\).

However, nearly all patients who experienced a marked response to these agents eventually developed an acquired resistance to TKI therapies, resulting in disease progression. In addition, some patients are intrinsically resistant to EGFR-TKIs even though their tumors harbor activating mutations of EGFR\(^{39,40}\).

There is still much to learn about the molecular causes of resistance to first-generation EGFR-TKI therapy. EGFR activates several well-characterized signal transduction pathways known to be implicated in cell survival and proliferation. If an EGFR mutant cancer can maintain the activity of the downstream signaling pathways in the presence of either gefitinib or erlotinib, this may lead to resistance. Indeed, different “escape pathways” such as MET amplification, HER2, BRAF, AXL, MAPK1, or PIK3CA signaling via point mutations or up-
regulation either alone or in conjunction with each other have been found in approximately 22% of tumor specimens from NSCLC patients with acquired resistance\(^{[41-43]}\).

Of note, several preclinical studies have shown that continued activation of AKT downstream signaling is sufficient to confer resistance against EGFR-TKIs. In addition, most, if not all, laboratory models of acquired resistance show continued activation of the AKT pathway despite TKI treatment. Thus, targeting AKT signaling may provide a rationale for novel therapeutic strategies to overcome EGFR-TKI resistance in NSCLC\(^{[44-48]}\). In this study, we present data showing that LC capilliposide can inhibit AKT activation and restore, at least partially, gefitinib sensitivity to NSCLC cells with acquired gefitinib resistance. In the presence of LC capilliposide, we
Recently, Chinese herbal medicine has attracted increasing attention due to its effects on multidrug resistance for cancer therapy\cite{3,4}. Our data presented here suggest a potential clinical impact of the therapeutic strategy with a regimen including LC capilliposide for NSCLC patients who failed in TKI therapy or have acquired TKI resistance. The combination treatment of gefitinib with LC capilliposide may also benefit NSCLC patients receiving TKI treatment as a first-line therapy. However, further studies are needed to reveal the detailed mechanisms and the epigenetics of NSCLC cells regarding the therapeutic effects of combining LC capilliposide with a first-generation TKI.

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Author contribution
Shi-rong ZHANG, Fan-zhu LI, and Sheng-lin MA designed the research; Shi-rong ZHANG, Ya-si XU, and Er JIN performed the research; Lu-cheng ZHU and Bing XIA analyzed the data; and Shi-rong ZHANG and Xu-feng CHEN wrote the paper.

Supplementary information
Supplementary information is available at the website of Acta Pharmacologica Sinica.

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Figure 5. LC capilliposide enhances tumor growth inhibition in response to gefitinib treatment in a PC-9-GR xenograft model. (A) The growth curves represented the average values of 8 mice in each group. Mean±SD. (B) Representative IHC images for the indicated proteins in the xenograft tumors. The scale bar represents 100 μm, and all of the images are to the same scale. P<0.05 vs Gefitinib single treatment group.
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