Cancer Management and Research

Original Research

Novel Hsp90 Inhibitor C086 Potently Inhibits Non-Small Cell Lung Cancer Cells As A Single Agent Or In Combination With Gefitinib

Liman Wang1–3,*
Yingjuan Fan1,*
Hanhao Mei1
Yang Liu1
Lianru Zhang4
Jianhua Xu1
Xuhui Huang2,3

1Institute of Materia Medica, School of Pharmacy, Fujian Provincial Key Laboratory of Natural Medicine, Pharmacology, Fujian Medical University, Fuzhou 350122, People’s Republic of China; 2Department of Pharmacy, Fujian Provincial Hospital Jinshan Branch/Fujian Provincial Hospital South Branch, Fuzhou 350028, People’s Republic of China; 3Provincial Clinical Medical College of Fujian Medical University, Fuzhou 350000, People’s Republic of China; 4State Key Laboratory of Cellular Stress Biology, School of Life Science, Xiamen University, Xiamen 361005, People’s Republic of China

*These authors contributed equally to this work

Purpose: Inhibition of heat shock protein 90 (Hsp90) can lead to degradation of multiple client proteins, which are involved in tumor progression. Elevated Hsp90 expression has been linked to poor prognosis in patients with non-small cell lung cancer (NSCLC). Discovery of effective drug is a promising strategy to improve patient survival. This study aims to investigate the synergistic antitumor mechanism of C086 combined with gefitinib in NSCLC cells in vitro.

Methods: The binding of C086, gefitinib, and the combinations to Hsp90 was characterized by fluorescence quenching experiments. The inhibition of A549 or NCI-H1975 cell proliferation and apoptosis by C086 and gefitinib as a single agent or in combinations were performed using CFSE staining assays, AnnexinV–APC/PI and Western blot.

Results: C086 alone or with gefitinib reduces proliferation and increases proapoptotic caspase activation of both wild-type and mutation NSCLC, with NCI-H1975 cells showing much greater sensitivity to C086 and the combinations than A549 cells. The combination of C086 and gefitinib showed synergistic reduction of EGFR expression and the downstream PI3K/Akt and Ras-Raf-Erk pathways enhanced suppression of Erk signaling.

Conclusion: C086 combined gefitinib has a good synergistic antitumor effect in vitro. Therefore, the combination of C086 and gefitinib may provide a new theoretical basis and ideas for the treatment of NSCLC patients.

Keywords: C086, Hsp90 inhibitor, EGFR, non-small cell lung cancer

Introduction

Lung cancer is the most common cause of cancer death throughout China and the world. Over 80% of lung cancer patients belong to the non-small cell lung cancer (NSCLC) group with a poor prognosis. The elevated overall epidermal growth factor receptor (EGFR) kinase activity, as a result of the increased amount and/or the gain-of-function mutations, plays a key role in the disease progression and cancer malignancy. These offer an effective therapeutic target to develop agents for NSCLC. Gefitinib, EGFR-tyrosine kinase inhibitor (TKI), is the approved therapy for NSCLC harboring EGFR with activating mutations. Unfortunately, those who respond to gefitinib at the early stages develop resistance because of the emergence of EGFR mutations or other genomic alterations with wild-type EGFR, including K-ras mutations. Circumventing the resistance to TKI is actually the most formidable challenge in treating NSCLC patients. Thus, identification of an effective treatment using rationalized combinations of agents is particularly promising.
Heat shock protein 90 (Hsp90) is a highly conserved molecular chaperone that plays an important role in the maturation and stabilization of over 200 oncogenic client proteins\(^1\)-\(^3\) and is considered to be an attractive target for cancer therapies.\(^4\)-\(^6\) Most Hsp90 client proteins, such as EGFR, Akt and C-Raf, are crucial for growth, differentiation and survival of tumors.\(^7\)-\(^9\) Increased HSP90 expression has been linked to worse prognosis in patients with NSCLC.\(^10\) To achieve synergistic treatment, Hsp90 inhibitor was chosen as another chemotherapeutic drug.

In our previous work,\(^11\)-\(^13\) we identified a novel potent Hsp90 inhibitor, 4-(4-hydroxy-3-methoxy-phenyl-methyl)curcumin (C086) (Figure 1A), which could inhibit cell cycle progression and induce cell apoptosis and antitumor activity by regulating various mechanisms in different cell types. Although the anticancer mechanisms of C086 and the antineoplastic activities of C086 combined with several clinical used antitumor drugs have been documented,\(^14\) the potential effects of C086 combined with gefitinib in NSCLC have not been investigated. In this study, two NSCLC cell lines A549 and NCI-H1975 were used to evaluate the properties of C086 alone and its combination with gefitinib on cell growth. Herein, we reveal potent antitumor activity of C086 as a single agent and in combination with gefitinib, which exhibited synergistic effects on inhibition of cell proliferation and enhanced apoptosis by modulating the EGFR protein kinase activity in NSCLC in vitro.

**Materials And Methods**

**Cell Lines, Plasmids And Reagents**
The human NSCLC cell lines A549 and NCI-H1975 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in...
RPMI-1640 media containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO2.

The bacterial strains and plasmids were obtained from the School of Life Science of Xiamen University, China. C086 (purity≥99%) was designed and synthesized by our laboratory (Figure 1B). C086 was dissolved in DMSO as a stock solution and diluted in culture media. Gefitinib was purchased from LC Laboratories (Woburn, MA, USA). Anti-Hsp90, anti-β-actin, anti-EGFR, anti-Ras, anti-C-Raf, anti-Akt, anti-P-Akt, anti-Mek, anti-P-Mek, anti-Erk½, anti-P-Erk, anti-C-Myc, anti-Bax, anti-Bcl-2 (an apoptosis suppression protein), caspase-8, cleaved caspase-8, and the Apoptosis Antibody Sampler Kit containing PARP, cleaved PARP, caspase-9, cleaved caspase-9, caspase-3, cleaved caspase-3, caspase-7, cleaved caspase-7 were purchased from Cell Signaling Technology, Inc (Danvers, MA, USA). Annexin-V-APC/PI Apoptosis Detection Kit was purchased from Nanjing Keygen Biotech Co. Ltd (Nanjing, China). Propidium iodide (PI) was obtained from Sigma Aldrich.

Fluorescence Measurements
Samples were excited at 280 nm and fluorescence intensities were recorded from 290 to 500 nm at 37°C using a Cary Eclipse spectrophluorometer (Varian, Palo Alto, CA, USA). The measurements were obtained with 2.0 mL of 5.0 μmol/L Hsp90 solution (10 mmol/L PBS buffer, pH 7.6) and successive additions of C086 and gefitinib as single agents or in combinations from 5 to 50 μmol/L. The binding interaction of C086 and gefitinib as single agents or in combinations with Hsp90 was expressed as a value of dissociation constants (Kd). All tests were performed in triplicate.

MTT Assays
Exponentially growing cells (1×104 cells/mL) were incubated in triplicate in a clear 96-well plates for 48 hrs at 37°C and treated with varying concentrations of C086 and gefitinib as single agents or in combinations dissolved in culture medium. Control cells were exposed to the medium and then 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Company, St Louis, MO, USA) solution (5 mg/mL) was added and incubated for 4 hrs. The dark blue formazan crystals were solubilized in DMSO and the optical density was detected at 570 nm with a spectrophotometer. Cell viability was assessed by the MTT assays. Growth inhibition rate was calculated according to mean values obtained from each concentration relative to control values, and the half maximal inhibitory concentrations (IC50) were calculated by PASW Statistics 18 (SPSS, Inc).

CFSE Staining Assays
Exponentially growing cells were resuspended in the CFSE (carboxyfluorescein diacetate succinimidyl ester) staining solution at 37°C for 10 mins. After washing with cold RPMI-1640 medium containing 10% heat-inactivated FBS, cells were grown in 6-well plates at a final concentration of 8×104 cells/well in the presence or absence of C086 and gefitinib as single agents or in combinations for 72 hrs at 37°C. The cells were resuspended in PBS and then analyzed by flow cytometry.

Apoptosis Assessment By Annexin-V Staining
The Annexin V-APC/PI Apoptosis Detection Kit (Nanjing, China) was used for the apoptosis assay according to the manufacturer’s instructions. Briefly, A549 cells and NCI-H1975 cells were treated with gefitinib, C086 or gefitinib plus C086 for 48 hrs. Subsequently, the NSCLC cells were resuspended in 100 μL of staining solution containing Annexin-V-APC/PI in buffer. After incubation at room temperature for 15 mins in the dark, cells were analyzed immediately using a flow cytometer (BD FACSCalibur, BD Biosciences, Franklin, NJ, USA). Annexin-V bound to cells that expressed phosphatidylserine on the outer layer of the cell membrane. Cells that stained positive for Annexin-V were scored as apoptotic cells.

Cell Cycle Assessment By PI Staining
Following the drug treatments, the cells were resuspended in PBS and fixed with 70% ethanol overnight at −20°C. After washing with cold PBS, cells were incubated with DNase-free RNase and propidium iodide (PI) staining at 37°C for 30 mins. Then, cells were analyzed immediately by flow cytometry.

Western Blot Analysis
The cancer cells were treated with compounds at indicated doses for 48 hrs, then washed with cold PBS and harvested with NP-40 lysis buffer containing protease inhibitor and phosphatase inhibitor. The cleared cell lysates were loaded and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins were transferred to a PVDF membrane (150 mA, 4°C).
for 1.5 hrs. The membranes were blocked in blocking buffer (1% BSA, Tris-HCl 20 mmol/L, pH 7.5, NaCl 150 mmol/L, and 0.05% Tween-20) for 1 hr at RT, followed by incubation with the relevant antibody overnight at 4°C. After binding of anti-rabbit peroxidase-conjugated secondary IgG antibodies, the membranes were visualized by ECL chemiluminescence according to the instructions of the manufacturer (Thermo Scientific, Waltham, MA, USA). The membranes were scanned on a Carestream Image Station System to visualize the bands.

Statistical Analysis
Statistical analysis of the data was performed with two-sided unpaired t-tests using the GraphPad software package for Windows (Prism version 7.0) and Origin 8.5 software. Values are reported as the mean values of triplicate or duplicate experiments. Differences were considered statistically significant at P<0.05.

Results

C086 Combined With Gefitinib Damages The Molecular Chaperone Functions Of Hsp90 In NSCLC Cells
The binding of C086, gefitinib and the combinations to Hsp90 was characterized by fluorescence quenching experiments. When Hsp90 was incubated with increasing concentrations of C086, gefitinib, or C086 plus gefitinib, the fluorescence intensity gradually decreased (Figure 1B). Using the equation previously described, the titration curves for Hsp90 yielded estimated dissociation constants (Kd). Kd values were determined to be 18.62±0.27 μmol/L, 8.52±0.25 μmol/L and 6.55±0.12 μmol/L, respectively (Figure 1C).

Treatment With C086 And Gefitinib In Combination Results In Synergistically Inhibit Proliferation Of NSCLC Cells
The feasibility of combining C086 with gefitinib in two NSCLC cell lines was assessed through a series of proliferation assays. First, we examined the single and additive effects on proliferation of A549 and NCI-H1975 cell lines. A549 cells were treated with C086, gefitinib, or C086 plus gefitinib ranging from 0.625 to 40 μmol/L, while NCI-H1975 cells were incubated with C086 and gefitinib as single agents or in combinations ranging from 0.625 to 40 μmol/L. The IC50 values of C086 for the A549 and NCI-H1975 cell lines were 5.13±1.08 and 2.97±0.12 μmol/L, respectively. Potential synergism between C086 and gefitinib in NSCLC cell lines was evaluated by the Chou–Talalay method, which is widely employed in drug combination and synergy quantification. The resulting combination index (CI) theorem offers quantitative definitions for additive effect (CI=1), synergism (CI<1) and antagonism (CI>1) in drug combinations. Effect of the optimal combinative concentrations obtained from Chou–Talalay method on the NSCLC cell viability was tested by MTT assay. Results from Figure 2A showed C086 is a more potent inhibitor of two NSCLC cells than gefitinib and significantly synergic growth inhibition by the drug combinations in vitro in A549 (CI=0.393) and NCI-H1975 (CI=0.304) cell lines compared with single drugs.

To gain a better understanding of the mechanism of inhibition, we determined the single and additive effects of treatment with C086 and gefitinib on the downstream PI3K/Akt and Ras-Raf-Erk signaling pathway. Exposure of A549 and NCI-H1975 cells to C086 or C086 plus gefitinib resulted in reduction of Hsp90 client proteins, such as EGFR, Raf, Akt, Erk and C-Myc. Of particular interest was the observed decrease in their phosphorylated forms in a similar manner (Figure 2B). The results indicated a synergistic blockade downstream signaling by combined treatment of C086 and gefitinib, with NCI-H1975 cells showing much greater sensitivity to C086 and the combinations than A549 cells.

C086 Cotreatment With Gefitinib Induces Cell Cycle Arrest In Both A549 And NCI-H1975 Cells
The inhibition of A549 or NCI-H1975 cell proliferation by C086 and gefitinib as a single agent or in combinations was also shown by CFSE staining assay (Figure 3A) in order to gain further insight into the effects of C086 with gefitinib on cell proliferation. These results indicated that both NSCLC cells were also sensitive to C086, gefitinib and the compound. Since the combination disrupts cell growth, we next studied the single and additive effects on the cell cycle distribution of A549 and NCI-H1975 cell lines. Significant G0/G1 phase cell cycle arrest was observed in conditions treated with gefitinib compared to controls, while treatment with C086 alone showed G2/M phase cell cycle arrest at 24 hrs. These results indicated that C086-treated cells were more efficient at inducing G2/M phase arrest both in A549 and in NCI-H1975 cells.
Interestingly, cells treated with the combination of C086 and gefitinib showed induced G2/M phase cell cycle arrest.

C086 Combined With Gefitinib Shows Synergistic Apoptotic Effect In NSCLC Cells

To determine whether the growth inhibition of A549 and NCI-H1975 cells by C086 with gefitinib is associated with the induction of apoptosis, we used AnnexinV–APC/PI staining and quantified the number of compound-induced apoptosis in A549 and NCI-H1975 cells. Consistent with the cellular proliferation assay results, apoptosis was induced in both A549 and NCI-H1975 cells (Figure 4A and B). Mechanism underlying the synergistic anticancer effects of the combination treatment with C086 and gefitinib was determined by measuring the levels of apoptosis-related proteins, including PARP, cleaved PARP, caspase-9, cleaved caspase-9, caspase-3, cleaved caspase-3, caspase-8, cleaved caspase-8, caspase-7, cleaved caspase-7, Bcl-2 and Bax in A549 and NCI-H1975 cells treated with gefitinib (5.0 and 2.5 μmol/L), C086 (5.0 and 2.5 μmol/L) or their combination for 48 hrs by performing Western blotting analysis. We found that C086 induces apoptosis through caspase pathway, and levels of cleaved PARP, cleaved caspase-9, cleaved caspase-3, cleaved caspase-8 and cleaved caspase-7 significantly increased in cells treated with the combination of C086 and gefitinib compared with those in cells treated with gefitinib or C086 alone (Figure 4C). Therefore, C086 in combination with gefitinib enhanced cell death which is an important feature in NSCLC cells.

Discussion

The main aims of drug combination are to achieve synergistic therapeutic effect, dose and toxicity reduction and to
minimize or delay the induction of drug resistance. Since curcumin displayed synergistic effects of several chemotherapeutic drugs, it has been suggested as an adjuvant for anticancer therapy. In the present work, we investigated the mechanisms of curcumin derivative, C086, in overcoming primary and acquired resistance to gefitinib in NSCLC with different EGFR status. We discovered that the combination of C086 and gefitinib displayed synergistic inhibition of proliferation and enhanced antitumor effects in two NSCLC cell lines (A549 and NCI-H1975 cells) in vitro. This was accompanied by more significant apoptotic cell death and augmented blockade of EGFR downstream signaling pathways as compared with monotherapy, irrespective of different resistance mechanisms.

It is now widely accepted that EGFR is a validated therapeutic target in NSCLC. Mutated EGFR and the mature, wild-type receptor that overexpress WT-EGFR are bona fide Hsp90 clients in cancer. We show here that two subtypes of NSCLC cells are inhibited in their proliferation by C086 as a single agent in a dose-
dependent manner. Furthermore, the combination of C086 with gefitinib exhibited synergistic enhancement of the antitumor activity (Figure 2A). C086 alone and in combination with gefitinib treatment inhibit the proliferation of A549 and NCI-H1975 cells through the downregulation of EGFR signal transduction pathway, such as PI3K/Akt and Ras-Raf-Erk signaling pathway, with NCI-H1975 cells showing much greater sensitivity to C086 and the combinations than A549 cells (Figure 2B). These results suggest that a more complete and sustained inhibition of EGFR can occur through inhibition of the Hsp90 chaperon pathway. Although the traditional Hsp90 inhibitors, such as geldanamycin (GA) and its derivative 17-allyamino-geldanamycin (17AAG), have exhibited potent anticancer effects, severe hepatotoxicity has prevented their clinical development. Treatment with C086 showed a more favorable safety profile during the whole experimental periods in the previous study. Combination therapy displayed significantly enhanced antitumor efficacy and reduced toxicity compared with gefitinib alone.

Moreover, the data presented here suggest that the capacity of C086 to potentiate the in vitro activity of gefitinib provides a compelling rationale for combining two agents as part of novel treatment strategies for NSCLC. Overall, the therapeutic benefit conferred by dual Hsp90/EGFR TKI blockade was conserved within both the mutant and WT-EGFR disease settings, thus providing new opportunities to target EGFR and overcome mechanisms of resistance across diverse groups of NSCLC patients.

**Conclusion**

As a novel Hsp90 inhibitor, C086 alone or in combination with gefitinib shows potent antitumor effects in NSCLC
cells in vitro. The combination of C086 and gefitinib displayed synergistic PI3K/Akt and Ras-Raf-Erk signaling depression, suggesting a promising strategy in treating NSCLC.

Acknowledgments
This work was funded by Fujian Provincial Health and Family Planning Commission of China (2016-1-33), the Startup Fund for Scientific Research, Fujian Medical University (2017XQ1133), the Joint Funds for the Innovation of Science and Technology, Fujian province, China (2016Y9059), the Projects of Industry-Academy Cooperation for Science, Technology of Fujian Province, China (2016Y4005) and the Open Research Fund of State Key Laboratory of Cellular Stress Biology, Xiamen University (SKLCSB2017KF003). Liman Wang and Yingjuan Fan are co-first authors for this study.

Disclosure
The authors report no conflicts of interest in this work.

References
The authors report no conflicts of interest in this work.


Wang et al

Cancer Management and Research 2019:11

8944

submit your manuscript | www.dovepress.com
Dovepress
Cancer Management and Research downloaded from https://www.dovepress.com by 54.70.41.11 on 10-Nov-2019
For personal use only.


