Enediyne lidamycin enhances the effect of epidermal growth factor receptor tyrosine kinase inhibitor, gefitinib, in epidermoid carcinoma A431 cells and lung carcinoma H460 cells

Hong Liu, Liang Li, Xing-Qi Li, Xiu-Jun Liu and Yong-Su Zhen

Gefitinib, a low-molecular-weight epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, is effective in a wide variety of tumor types. Preclinical studies have shown potentiated antitumor efficacies of this agent in combination with chemotherapy or radiotherapy. The antitumor antibiotic lidamycin (LDM) showed extremely potent cytotoxicity *in vitro* and marked therapeutic effect *in vivo*. In this report, the cytotoxic and biochemical activity of LDM and gefitinib on human epidermoid carcinoma A431 cells and human large cell lung cancer H460 cells as a single agent or in combination has been evaluated. In the MTT assay, LDM showed much more potent cytotoxicity than gefitinib to both cell lines. A431 cells with a highly EGFR-expressing level were more sensitive to gefitinib than H460 cells, which expressed EGFR at an intermediate level. LDM plus gefitinib showed potentiation of antiproliferative activity and apoptosis induction, which were associated with downregulation of EGFR signaling pathway and nuclear factor-kappa B expression, and the increase of cleaved poly (adenosine diphosphate-ribose) polymerase in the two cell lines, although to a lesser degree in H460 cells. Combined treatment induced G_1 phase arrest similar to that of gefitinib alone in A431 cells and intensified G_2/M phase accumulation in H460 cells. The above results indicate that LDM potentiates the effects of gefitinib in both gefitinib sensitive and less sensitive cells in association with enhanced inhibition of EGFR-dependent signaling. *Anti-Cancer Drugs* 20:41–49

Anti-Cancer Drugs 2009, **20**:41–49

Keywords: apoptosis, combination therapy, enediyne, epidermoid carcinoma, gefitinib, lidamycin, lung carcinoma

Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

Correspondence to Yong-su Zhen, #1 Tian Tan Xi Li, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China
Tel: +86 10 8315 8065; fax: +86 10 8315 8065;
e-mail: zhenys@public.bta.net.cn

Received 25 July 2008 **Revised form accepted** 7 September 2008

Introduction

The epidermal growth factor receptor (EGFR), a member of the ErbB family of receptors, is abnormally activated in many epithelial tumors. Activation of the EGFR protein tyrosine kinase results in the recruitment and phosphorylation of several intracellular substrates. Two major downstream signaling routes of the EGFR family are the Ras–Raf–MEK-ERK pathway and the PI3K/Akt pathway. ERK1/2 regulates the transcription of molecules that are linked to cell proliferation, survival, and transformation. Akt signals trigger a cascade of responses from cell growth and proliferation to survival and motility [1].

The EGFR signaling pathway contributes to a number of processes important in cancer development and progression such as proliferation, apoptosis, angiogenesis, and metastatic tumor spread. Accordingly, targeting the EGFR represents an appealing molecular target-based approach to promote selective cancer therapy. Gefitinib is an orally active, selective reversible inhibitor that prevents autophosphorylation of EGFR by physical interaction with its intracellular kinase domain. In preclinical studies with cultured cell lines and human tumor xenografts, gefitinib as a single-agent therapy exerts growth inhibition in a wide variety of tumor types including lung, prostate, breast, colon, ovarian, small cells, and non-small cell lung cancers (NSCLC) with various expression levels of EGFR [2,3].

Lidamycin (LDM, also called C-1027), an enediyne antitumor antibiotic, is produced by *Streptomyces globisporus C*-1027 [4]. LDM contains an acid protein of 110 amino acid residues and a chromophore of novel enediyne structure, with the former serving as a protecting protein whereas the latter as an active component to damage DNA [5]. LDM showed extremely potent cytotoxicity toward culture cancer cells and markedly inhibited the growth of transplantable tumors in mice and human cancer xenografts in nude mice [6–8]. LDM is also highly active in multidrug-resistant cancer cells [9]. In addition, LDM is a new potent antiangiogenesis agent with markedly antimetastatic activity [6]. The potent efficacy of LDM was ascribing to its DNA strand-scission activity [10]. LDM is currently being evaluated in phase II

0959-4973 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e328318292c

clinical trials as a potential chemotherapeutic agent in China.

In this study, we have used two human epithelial cancer cell lines with functional EGFR-dependent autocrine pathways for defining the differential effects of a combination of the anti-EGFR agent gefitinib and enediyne LDM on cell growth, cell cycle distribution, induction of apoptosis, and inhibition of the activation of EGFR signal pathway. These findings suggest that LDM potentiates the effects of gefitinib in both gefitinib sensitive and less sensitive cells by enhanced down-regulation of the phosphorylation of EGFR signal molecules in combination treatment.

Materials and methods

Chemicals and reagents

LDM (provided by Professor Lian-fang Jin of our institute) was prepared into 10 μmol/l with 0.9% NaCl solution and stored at −80°C before use. The EGFR-TKI gefitinib (AstraZeneca Pharmaceuticals, Macclesfield, UK) was diluted in dimethylsulfoxide (DMSO) to a stock concentration of 20 mmol/l. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) and DMSO were purchased from Sigma (St Louis, Missouri, USA). All other chemicals were of standard analytical grade.

Cell culture

Human epidermoid carcinoma A431 cells and human large-cell lung carcinoma H460 cells were obtained from the Cell Center of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College. A431 and H460 cells were cultured, respectively, in DMEM and RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mmol/l glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37°C in a humidified 95% air and 5% carbon dioxide atmosphere.

Cell proliferation assay

Cells were seeded in 96-well plates (Costar, Cambridge, Massachusetts, USA) with 3000–6000 cells/well. After overnight incubation, triplicate wells were treated with varying concentrations of LDM and gefitinib for 48 h. Then, 20 μl MTT solutions (5 mg/ml in PBS) were added to each well and incubated for 4 h at 37°C. The MTT formazan was dissolved in 150 μl DMSO and absorbance was measured by a Microplate Reader (Multiskan MK3; Thermo Labsystem, USA) at a wavelength of 570 nm. For the evaluation of the antiproliferative effects of the combined treatment with LDM and gefitinib, cells were pretreated with LDM for 8 h before exposure to gefitinib. Cell viability was determined after 72 h treatment. The results from the combination assays were analyzed using the isobologram combination index (CI) method of Chou and Talalay [11]. All the experiments were performed in triplicate.

FITC–annexin V/PI apoptosis assay

Cells were harvested and resuspended in a 200 μl binding buffer. Then 10 μl FITC-labeled enhanced annexin V (Baosai Biotechnology Ltd, Beijing, China) and 100 ng propidium iodide were added. Upon incubation in the dark (15 min, room temperature or 30 min at 4°C), the samples were diluted with 300 μl binding buffer. The percentage of annexin V-positive cells was counted on FACScan flow cytometer (Becton Dickinson, New Jersey, USA) using WinMDI/PC software. For the evaluation of the apoptotic effects of the combined treatment with LDM and gefitinib, cells were treated in the sequence of LDM for 8 h followed by gefitinib up to 48 h.

Cell cycle analysis

Cells were treated in the sequence of LDM for 8 h followed by gefitinib up to 48 h. Floating and adherent cells were harvested and centrifuged at 600*g* for 5 min. Then cells were fixed in ice-cold 70% ethanol and stored at −20°C for 24 h before analysis. For cell cycle analysis, cells were washed twice in PBS and stained with 50 μg/ml propidium iodide and 200 μg/ml RNase A for 30 min. The samples were analyzed on a fluorescence-activated cell sorter (EPICS XL; Beckman Coulter, Hialeah, Florida, USA).

Western blot analysis

Cells were treated in the sequence of LDM for 8 h followed by gefitinib up to 48 h. Cell culture monolayers were washed twice with ice-cold PBS and lysed with lysis buffer containing 50 mmol/l Tris–HCl, pH 7.5, 150 mmol/l NaCl, 2 mmol/l EDTA, 2 mmol/l EGTA, 1 mmol/l dithiothreitol, 1% Nonidet P-40, 0.1% SDS, protease inhibitors (1 mmol/l PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin) and phosphatase inhibitors (20 mmol/l β-glycerophosphate, 50 mmol/l NaF and 1 mmol/l Na_3VO_4). Protein concentration was determined by the BCA protein assay (Pierce Chemical Co., Rockford, Illinois, USA). An equal amount of protein samples were loaded on SDS–PAGE and transferred to PVDF (Millipore, Bedford, Massachusetts, USA). Membranes were then incubated in the blocking solution (5% bovine serum albumin in 20 mmol/l Tris–HCl, 150 mmol/l NaCl, 0.1% Tween-20) (TBS-T), followed by incubation with the indicated antibodies at 4°C overnight. The membranes were then washed in TBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Antibody detection was performed by an enhanced chemiluminescence reaction (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). To control for protein loading, the blot was stripped and rehybridized with the β-actin antibody. The following antibodies were used: antiphospho-EGFR, anti-EGFR, antiphospho-Akt (Ser473),

anti-Akt, antiphospho-ERK, anti-ERK, anti-nuclear factor-kappa B and anticleaved poly (adenosine diphosphate-ribose) polymerase (PARP) (all from Cell Signaling Technology, USA).

Statistical analysis

The data of at least three comparable experiments were expressed as the mean ± SEM. For all of the measurements, one-way analysis of variance followed by paired *t*-test was used to assess statistical significance of difference between different treatment groups. Significance was defined as $P < 0.05$.

Results

Effects of gefitinib and lidamycin on cell proliferation

Dose–response growth inhibitory effects were observed in antiproliferative assays. From Fig. 1, a remarkable difference in sensitivity to LDM and gefitinib was found between the two cell lines. Compared in terms of IC_{50}, LDM was more than 1000-fold more potent in cytotoxicity than gefitinib. The levels of EGFR seemed to be associated with the sensitivity to gefitinib, as the potent inhibition (IC_{50}, 0.28 ± 0.03 μmol/l) was observed in highly EGFR-expressing A431 cells whereas the moderate EGFR-expressing H460 cells were relatively resistant (IC_{50}, 19.57 ± 6.6 μmol/l). In contrast, the highly EGFR-expressing A431 cells seemed to be less sensitive to LDM (IC_{50} for A431 cells: $1.12 \times 10^{-10} \pm 1.10 \times 10^{-10}$ mol/l; for H460 cells: $3.65 \times 10^{-12} \pm 2.94 \times 10^{-12}$ mol/l).

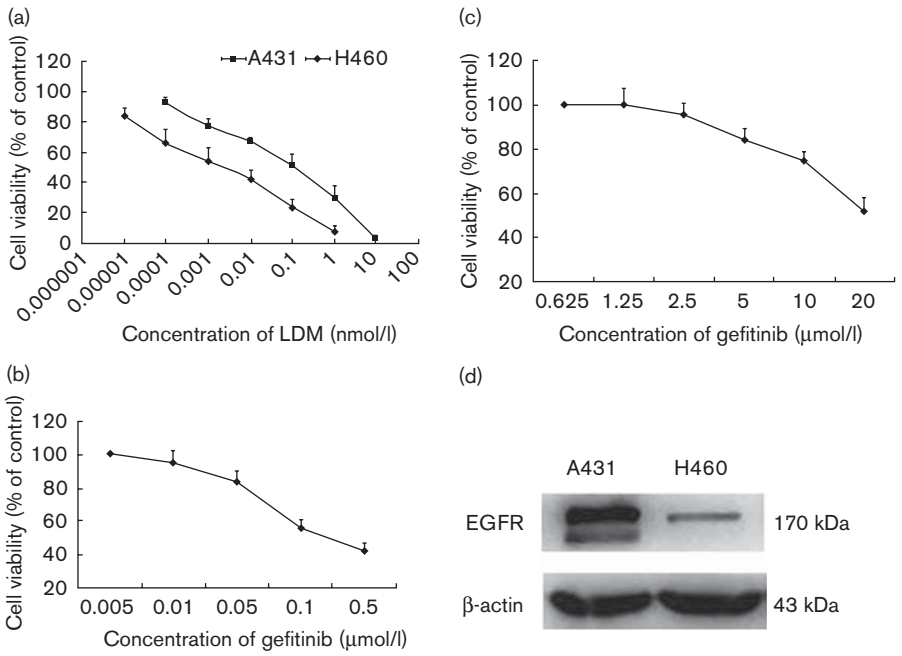
Enhanced antiproliferative effects of the combined treatment

To evaluate the nature of the interaction between gefitinib and LDM (additive or synergistic), combination analyses were performed according to the Chou and Talalay method [11]. As Fig. 2 illustrates, slight-to-moderate synergistic growth inhibitory effects (CI < 1) at a majority of combinations were found in the two cell lines. Strong synergistic effects were achieved in certain combination doses. The CI was less than 0.70 at the combination dose of 5 μmol/l gefitinib plus 0.1 nmol/l LDM in H460 cells, and 0.1 μmol/l gefitinib plus 0.01 nmol/l LDM in A431 cells.

Induction of apoptosis by gefitinib and lidamycin

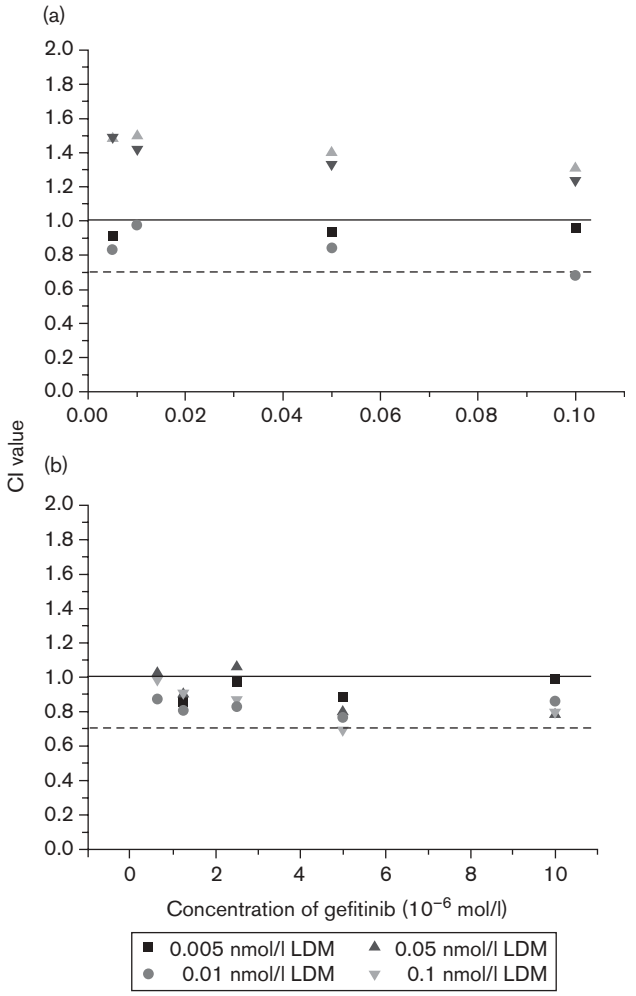
Flow cytometry combined with FITC–annexin V/PI staining showed that the percentage of apoptotic cells increased dose-dependently in the two cell lines. LDM showed potent apoptosis induction effects on both A431 cells and H460 cells as the apoptotic cells induced by 2 nmol/l LDM reached $69.87 \pm 3.29\%$ ($P < 0.001$) and $63.22 \pm 1.39\%$ ($P < 0.001$), respectively. As shown in

Fig. 1



Effect of LDM and gefitinib on cell viability in human epidermoid carcinoma A431 cells and human large cell lung cancer H460 cells. (a) A431 cells and H460 cells were incubated with various concentrations of LDM for 48 h. (b) A431 cells and (c) H460 cells were incubated with various concentrations of gefitinib for 48 h. At the end of incubation, the cell survival rates were determined by MTT methods. Cell viability is expressed as the percentage of cell survival compared with the control. Data are from three independent experiments. (d) The levels of EGFR expression in A431 cells and H460 cells by western blot analysis. Equal loading was determined by stripping the membranes and reblotting with an antibody to β-actin. EGFR, epidermal growth factor receptor; LDM, lidamycin.

Fig. 2



Cell viability assay of A431 cells (a) and H460 cells (b) in response to LDM or gefitinib alone or in combination. Cells were pretreated with LDM for 8 h followed by gefitinib up to 72 h. CI is a quantitative measure of the degree of interaction between different drugs. When CI values between 1 and 0.7 indicate slight to moderate synergism; CI values of 0.7 to 0.3, strong synergism. CI, combination index; LDM, lidamycin.

Fig. 3, the A431 cell line was more sensitive to gefitinib. There occurred $30.70 \pm 1.69\%$ of apoptotic cells ($P < 0.01$) as A431 cells exposed to 1 μmol/l gefitinib whereas only $13.60 \pm 1.37\%$ ($P < 0.05$) apoptotic cells were induced by 20 μmol/l gefitinib in H460 cells.

Lidamycin potentiated the apoptosis-inducing effect of gefitinib

FITC–annexin V/PI analysis showed that the combination treatment enhanced the induction of apoptosis in A431 cells and in H460 cells. As shown in Table 1, 0.1 μmol/l gefitinib plus 0.01 nmol/l LDM induced apoptosis in 31.87% of A431 cells, whereas the same doses of gefitinib and LDM given alone resulted in apoptosis in only 12.79 and 6.71% of cells, respectively.

Table 1 LDM potentiated the apoptosis-inducing effect of gefitinib in A431 cells and H460 cells

Drugs	Apoptotic cells (%)
A	
Control	3.96
LDM (0.01 nmol/l)	6.71
Gefitinib (0.1 μmol/l)	12.79
LDM (0.01 nmol/l) + gefitinib (0.1 μmol/l)	31.87
Gefitinib (1 μmol/l)	31.89
LDM (0.01 nmol/l) + gefitinib (1 μmol/l)	52.41
B	
Control	5.34
LDM (0.5 nmol/l)	42.88
Gefitinib (10 μmol/l)	7.05
LDM (0.5 nmol/l) + gefitinib (10 μmol/l)	46.31
Gefitinib (20 μmol/l)	12.63
LDM (0.5 nmol/l) + gefitinib (20 μmol/l)	53.85

A431 cells (A) and H460 cells (B) were treated with LDM or gefitinib alone or in combination for 48 h. Analyses of apoptosis were performed by FITC–annexin V/PI apoptosis assay, as described in Materials and methods LDM, lidamycin.

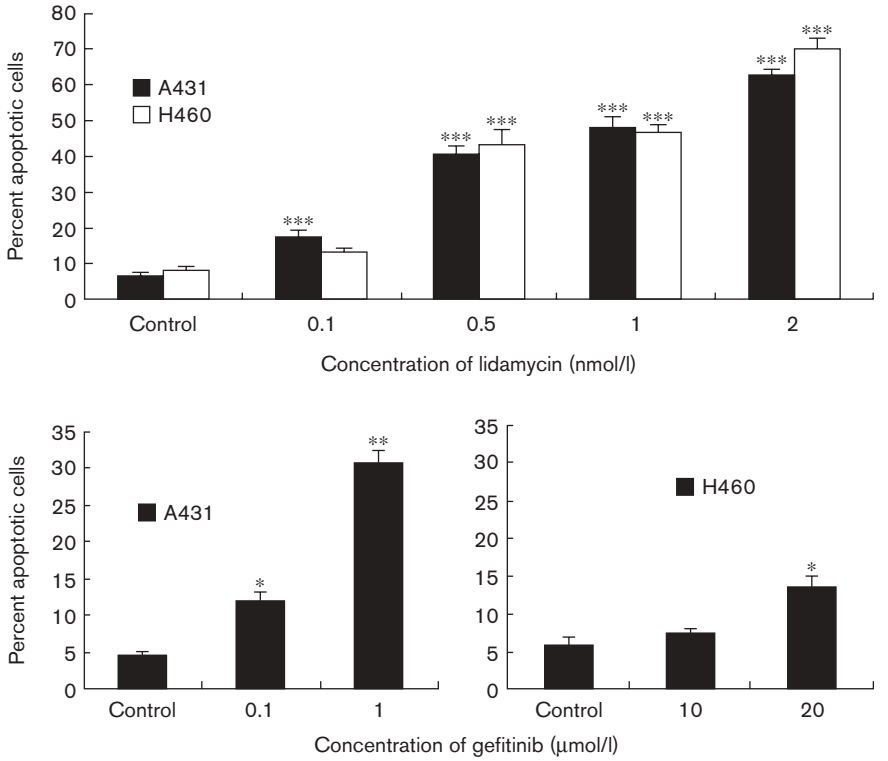
Similarly, at a higher dose level combination (1 μmol/l gefitinib plus 0.01 nmol/l LDM) the apoptotic rate was 52.41%, clearly higher than that of the 31.89 and 6.71% apoptosis with single-agent gefitinib and LDM, respectively. Only a small proportion of the H460 cells had undergone apoptosis even if a high dose of gefitinib was used. However, LDM potentiated the apoptosis-inducing effect of gefitinib at certain doses. As shown, 20 μmol/l gefitinib plus 0.5 nmol/l LDM induced apoptosis in 53.85% of cells, whereas the same dose of gefitinib and LDM given alone resulted in apoptosis in only 12.63 and 42.88% of cells, respectively.

Western blot analysis was used to detect the cleavage of PARP, an indicator of caspase-mediated apoptosis. The 89-kDa cleaved fragment of PARP increased dose-dependently in A431 cells exposed to gefitinib. Enhanced levels of cleaved PARP appeared after combination treatment (Fig. 4). A slightly enhanced effect was observed in H460 cells. Nevertheless, greater down-regulation of the antiapoptotic molecules nuclear factor-kappa B in the two cell lines was detected in the case of combined treatment.

Effects of gefitinib or lidamycin alone or in combination on cell cycle progression

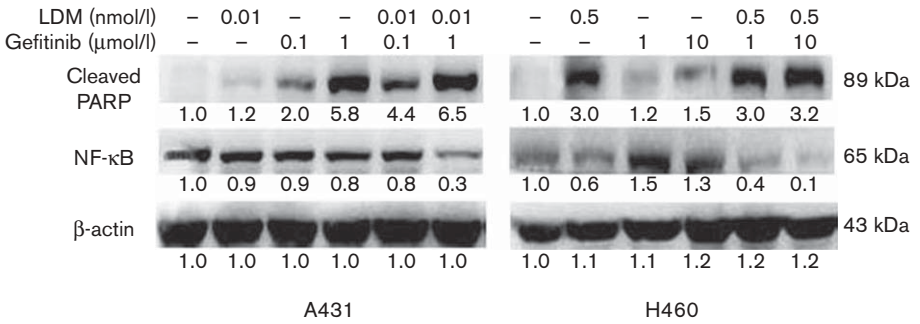
Cell cycle progression was evaluated after PI staining and FACS analysis. An increase of cells in the G_1 phase by 6.53–25.89% with a corresponding decrease in cells in the S and G_2/M phases was observed in A431 cells upon treatment with 0.1 and 1 μmol/l gefitinib. LDM at 0.01 nmol/l had little effect, either alone or in combination with gefitinib, on cell cycle progression (Fig. 5). In contrast, no evident change in cell cycle distribution was detected in H460 cells after exposure to gefitinib at indicated doses. However, combined treatment increased the G_2/M phase cells by 40.66–41.96% compared with single gefitinib treatment.

Fig. 3



Induction of cell apoptosis by lidamycin (LDM) and gefitinib in A431 cells and H460 cells. Cells were incubated with various concentrations of LDM and gefitinib for 48 h. Apoptosis was determined by the FITC–annexin V/PI apoptosis assay. Data represent the means ± SEM of three independent experiments. *$P<0.05$ vs. the control, **$P<0.01$ vs. the control, ***$P<0.001$ vs. the control.

Fig. 4

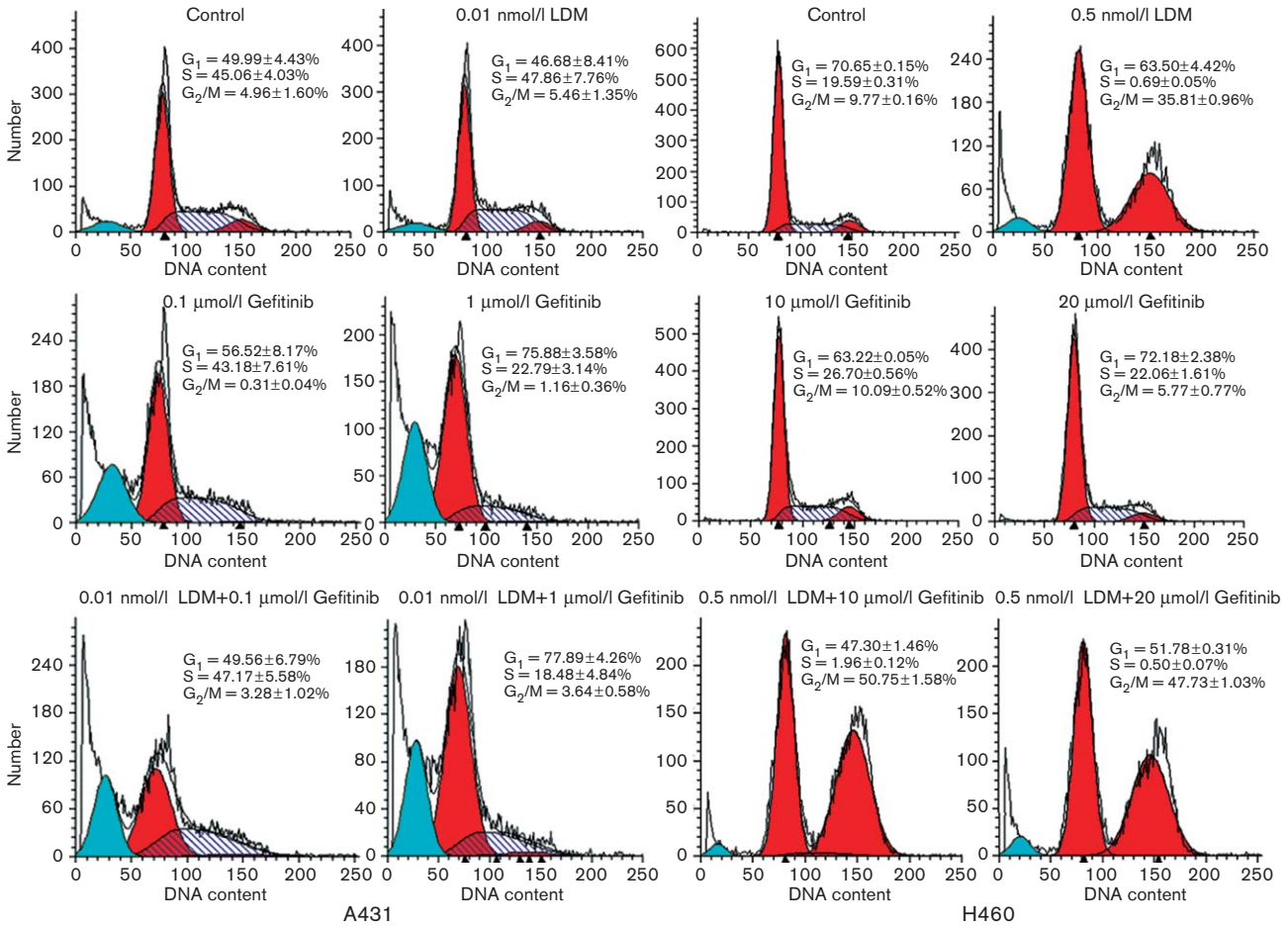


Effects on apoptosis-associated proteins of LDM or gefitinib or in combination in A431 cells and H460 cells. Equal loading was determined by stripping the membranes and reblotting with an antibody to β-actin. The figure is representative of three independent experiments. Values indicate the relative density of the bands. LDM, lidamycin; NF-κB, nuclear factor-kappa B; PARP, poly (adenosine diphosphate-ribose) polymerase.

Suppression of epidermal growth factor receptor signaling pathways by combined treatment

The signaling pathways induced by activated EGFR include the Ras/MEK/ERK and the Ras/PI3K/Akt pathways, both playing a significant role in the mitogenic and cell survival responses mediated by this receptor. As shown in Fig. 6, western blot analysis identified that phosphorylation of EGFR, ERK, and Akt was decreased in gefitinib-treated A431 cells, which is in agreement with previous observations by others in these cells [12]. A potentiated decrease in the levels of phosphorylation of each marker was observed in a combination of gefitinib and LDM at two dose combinations [gefitinib (μmol/l)/LDM (nmol/l): 0.1/0.01 and 1/0.01] in A431

Fig. 5



Cell cycle analysis of cells treated with lidamycin (LDM) or gefitinib alone or in combination. A431 cells and H460 cells were stained with PI after a 48 h exposure to different concentrations of single agent or in combination. Percentages of the total cell population in the different phases of the cell cycle were determined and included. Representative results of at least three experiments are shown.

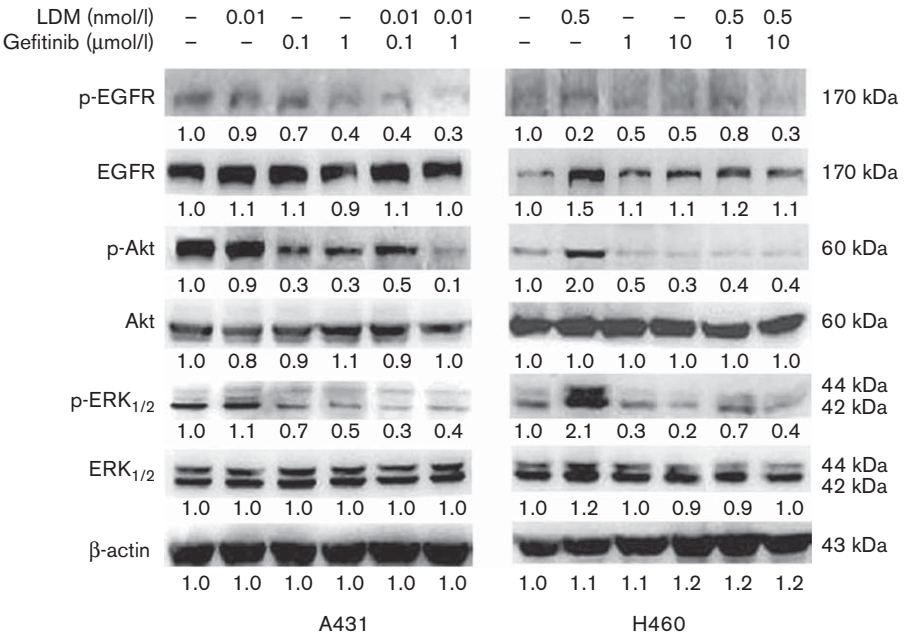
cells. Gefitinib alone also resulted in a reduction in phosphorylation of EGFR, ERK, and Akt in H460 cells at indicated doses. The combination of 0.5 nmol/l LDM with 1 or 10 μmol/l gefitinib slightly decreased the level of p-Akt and p-EGFR, respectively, as compared with each single agent in H460 cells. Notably, the EGFR expression level basically remained unchanged. These results suggest that the inhibition of EGFR signaling achieved with the combination of LDM with gefitinib can be augmented beyond that achieved using either agent alone and this inhibition correlated well with the growth inhibitory effects observed in cell proliferation and apoptosis assays.

Discussion

Gefitinib, a selective reversible EGFR inhibitor that blocks signal transduction pathways implicated in the proliferation and survival of tumor cells, is effective in a subset of NSCLC patients. However, many patients do not experience favorable responses to gefitinib. Clinical resistance to gefitinib highlights the need for improved therapeutic strategies [13,14]. The possibility of combining conventional cytotoxic drugs with EGFR TKIs has generated wide interest. Various preclinical studies have demonstrated the additive or even synergistic antitumor activity of gefitinib in combination with other anticancer therapies, particularly chemotherapeutic agents and radiation [15,16]. Relevant clinical issues are still open for the optimal use of gefitinib in combination with cytotoxic drugs.

LDM is currently being evaluated in phase II clinical trials as a potential chemotherapeutic agent in China. LDM induces unusual DNA damage of double-strand breaks [17]. It can induce apoptosis or mitotic cell death and alter cell cycle progression in many cancer cells [8,18,19]. LDM showed extremely potent cytotoxicity toward a wide variety of cancer cells. So far, there are no

Fig. 6



Effects on EGFR downstream signaling molecules of LDM or gefitinib or in combination in A431 cells and H460 cells. Cells were treated with LDM or gefitinib alone or in combination for 48 h. Equal amounts of protein were separated by SDS–PAGE and probed with the antibodies indicated. Equal loading was determined by stripping the membranes and reblotting with an antibody to β-actin. The figure is representative of three independent experiments. Values indicate the relative density of the bands. EGFR, epidermal growth factor receptor; LDM, lidamycin.

reports describing the combined efficacy of gefitinib with LDM. In this study, the gefitinib-sensitive A431 cells and gefitinib-resistant H460 cells were chosen for evaluating whether LDM can potentiate cell sensitivity to gefitinib in both cell lines.

In this study, we have shown potentiation of the antiproliferative activity of gefitinib by combined treatment with LDM. This was accompanied by the potentiation of cell cycle arrest and significant increase in cancer cell apoptosis with combination treatment. The cellular responses to treatment with gefitinib were consistent with the earlier findings [12,20–22] that A431 cells were more sensitive to gefitinib than H460 cells; furthermore, the augmented effects of gefitinib by LDM were much potent in A431 cells than in H460 cells. These results indicate that the synergic effect is, at least in part, cell type-dependent and related to the cell sensitivity to gefitinib.

According to unequivocal evidence presented in the literature, somatic mutations in the EGFR tyrosine kinase domain and *EGFR* gene amplification and protein overexpression have been associated with sensitivity to EGFR-targeted therapy [13,23–25]. In fact, the gefitinib-sensitive A431 cell line is known to harbor about 30 copies of the *egfr* gene within its genome, resulting in excessive EGFR protein expression [26,27]. The NSCLC H460 cell line used in this study expressed a moderate level of EGFR when compared with A431 cell line. This may explain the differential effects of gefitinib on the two cell lines.

We explored the potential mechanisms underlying the observed enhancement effect of the combination of gefitinib and LDM. Earlier studies have shown that gefitinib has varying effects on tumor cells including cell cycle arrest, increase of apoptosis and reduction in cell proliferation [12,15]. The effect of gefitinib was cytostatic, but higher doses increased apoptotic cell death. Recent studies suggest that gefitinib is able to induce apoptosis in cells of various histogenous cancers [12,28–30] and that it synergistically promotes cytotoxic effects of conventional chemotherapeutic agents such as paclitaxel in various cancers [12]. The A431 cell line that highly expresses EGFR is dependent on EGFR signaling for survival. Inactivation of the EGFR tyrosine kinase enzyme by gefitinib causes the cessation of growth and induction of mostly apoptotic cell death in A431 cells [31–33]. In addition, this study suggests that LDM and gefitinib given together exert an intensified blockade on EGFR activation and downstream signaling, which resulted in a greater degree of growth inhibition and apoptosis induction in A431 cells. In other words, the cooperative effects are because of the enhanced decrease in the activation of EGFR signal pathway with combined treatment in A431 cells.

As gefitinib alone showed a limited effect in inhibiting growth and inducing apoptosis of H460 cells, we hypothesized that a combination approach of LDM and gefitinib might be useful in augmenting growth suppression and apoptosis. The results suggested that LDM could accentuate the antiproliferative and apoptosis-inducing effect of gefitinib at certain combination doses. Furthermore, slightly potentiated inhibition of p-EGFR and p-Akt expression was observed with a combination treatment in H460 cells. Interestingly, increases in p-EGFR, p-ERK, p-Akt were observed in H460 cells after treatment with LDM. As reports suggest, similar results were obtained when other cytotoxic drugs (cisplatin or Taxol) were combined with gefitinib in NSCLC and colorectal cancer cell lines [34,35]. Their results suggested that increase of EGFR activity following drug treatment seems to determine the synergistic interaction between gefitinib and chemotherapy. Chemotherapy-induced activation of EGFR renders the cancer cells more sensitive to EGFR inhibition. In this study, enhanced EGFR activation by LDM may sensitize the resistant H460 cells to gefitinib. The cooperative interactions may be attributed to the suppression of EGFR and Akt signaling pathway in combination treatment, although further study is needed to clarify the details.

The above results showed that the levels of EGFR were associated with sensitivity to gefitinib in the two cell lines. Furthermore, the combined effect is related to diverse cell sensitivity to gefitinib, as more potent augmented effects were observed in gefitinib-sensitive A431 cells. These findings suggest that modulation of EGFR-mediated signal transduction cascades by LDM potentiates the antiproliferation and apoptosis induction effect of gefitinib. The enhanced effect in gefitinib-resistant H460 cells is especially promising. These findings suggest that such a combination strategy might represent a novel and promising approach for cancer patients, particularly for those patients who have developed resistance to EGFR inhibitor therapy. Further studies with *in vivo* tumor models are necessary to confirm the effectiveness of this novel therapeutic strategy.

Acknowledgement

This study was supported by the National High Technology Research and Development 863 Program of China (2004AA2Z3950).

References

1 Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell* 2000; **103**:211–225.
2 Ciardiello F, Tortora G. A novel approach in the treatment of cancer: targeting the epidermal growth factor receptor. *Clin Cancer Res* 2001; **7**:2958–2970.
3 Ranson M. ZD1839 (Iressa): for more than just non-small cell lung cancer. *Oncologist* 2002; **7** (**Suppl 4**):16–24.
4 Hu JL, Xue YC, Xie MY, Zhang R. A new macromolecular antitumor antibiotic, C-1027. I. Discovery, taxonomy of producing organism, fermentation and biological activity. *J Antibiot* 1988; **41**:1575–1579.
5 Tanaka T, Fukuda-Ishisaka S, Hirama M, Otani T. Solution structures of C-1027 apoprotein and its complex with the aromatized chromophore. *J Mol Biol* 2001; **309**:267–283.
6 Zhen HY, Xue Y, Zhen YS. Inhibition of angiogenesis by antitumor antibiotic C-1027 and its effect on tumor metastasis. *Natl Med J China* 1997; **77**:657–660.
7 Huang YH, Shang BY, Zhen YS. Antitumor efficacy of lidamycin on hepatoma and active moiety of its molecule. *World J Gastroenterlo* 2005; **11**:3980–3984.
8 Chen J, Ouyang ZG, Zhang SH, Zhen YS. Down-regulation of the nuclear factor-κB by lidamycin in association with inducing apoptosis in human pancreatic cancer cells and inhibiting xenograft growth. *Oncol Rep* 2007; **17**:1445–1451.
9 Shi YK, Wu SY, Huang YH, Zhen YS. Chemosensitivity of mdr1 gene overexpressed multidrug resistant cancer cells to lidamycin. *Acta Pharm Sin* 2006; **41**:1146–1151.
10 Sugiura Y, Matsumoto T. Some characteristics of DNA strand scission by macromolecular antitumor antibiotic C-1027 containing a novel enediyne chromophore. *Biochemistry* 1993; **32**:5548–5553.
11 Chou TC, Talalay P. Analysis of combined drug effects: a new look at a very old problem. *Trends Pharmacol Sci* 1983; **4**:450–454.
12 Janmaat ML, Kruyt FA, Rodriguez JA, Giaccone G. Response to epidermal growth factor receptor inhibitors in non-small cell lung cancer cells: limited antiproliferative effects and absence of apoptosis associated with persistent activity of extracellular signal-regulated kinase or Akt kinase pathways. *Clin Can Res* 2003; **9**:2316–2326.
13 Wheatley-Price P, Shepherd FA. Epidermal growth factor receptor inhibitors in the treatment of lung cancer: reality and hopes. *Curr Opin Oncol* 2008; **20**:162–175.
14 Lu Y, Liang K, Li X, Fan Z. Responses of cancer cells with wild-type or tyrosine kinase domain-mutated epidermal growth factor receptor (EGFR) to EGFR-targeted therapy are linked to downregulation of hypoxia-inducible factor-1alpha. *Mol Cancer* 2007; **6**:63.
15 Ciardiello F, Caputo R, Bianco R, Damiano V, Pomatico G, De PS, *et al*. Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. *Clin Cancer Res* 2000; **6**:2053–2063.
16 Sirotnak FM, Zakowski MF, Miller VA, Scher HI, Kris MG. Efficacy of cytotoxic agents against human tumor xenografts is markedly enhanced by coadministration of ZD1839 (Iressa), an inhibitor of EGFR tyrosine kinase. *Clin Cancer Res* 2000; **6**:4885–4892.
17 Kennedy DR, Beerman TA. The radiomimetic enediyne C-1027 induces unusual DNA damage responses to double-strand breaks. *Biochemistry* 2006; **45**:3747–3754.
18 Jiang B, Li DD, Zhen YS. Induction of apoptosis by enediyne antitumor antibiotic C1027 in HL-60 human promyelocytic leukemia cells. *Biochem Biophys Res Commun* 1995; **208**:238–244.
19 He QY, Liang YY, Wang DS, Li DD. Characteristics of mitotic cell death induced by enediyne antibiotic lidamycin in human epithelial tumor cells. *Int J Oncol* 2002; **20**:261–266.
20 Albanell J, Codony-Servat J, Rojo F, Del CJ, Sauleda S, Anido J, *et al*. Activated extracellular signal-regulated kinases: association with epidermal growth factor receptor/transforming growth factor á expression in head and neck squamous carcinoma and inhibition by anti-epidermal growth factor receptor treatments. *Cancer Res* 2001; **61**:6500–6510.
21 Anderson NG, Ahmad T, Chan K, Dobson R, Bundred NJ. ZD1839 (Iressa), a novel epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, potently inhibits the growth of EGFR-positive cancer cell lines with or without erbB2 overexpression. *Int J Cancer* 2001; **94**:774–782.
22 Moasser MM, Basso A, Averbuch SD, Rosen N. The tyrosine kinase inhibitor ZD1839 ('Iressa') inhibits HER2-driven signaling and suppresses the growth of HER2-overexpressing tumor cells. *Cancer Res* 2001; **61**:7184–7188.
23 Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, *et al*. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004; **350**:2129–2139.
24 Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, *et al*. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004; **304**:1497–1500.
25 Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, *et al*. EGF receptor gene mutations are common in lung cancers from 'never smokers'

and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci U S A* 2004; **101**:13306–13311.

26 Wikstrand CJ, McLendon RE, Friedman AH, Bigner DD. Cell surface localization and density of the tumor-associated variant of the epidermal growth factor receptor, EGFRvIII. *Cancer Res* 1997; **57**:4130–4140.

27 Merlino GT, Xu YH, Ishii S, Clark AJ, Semba K, Toyoshima K, *et al*. Amplification and enhanced expression of the epidermal growth factor receptor gene in A431 human carcinoma cells. *Science* 1984; **224**: 417–419.

28 Solomon B, Hagekyriakou J, Trivett MK, Stacker SA, McArthur GA, Cullinane C. EGFR blockade with ZD1839 ('Iressa') potentiates the antitumor effects of single and multiple fractions of ionizing radiation in human A431 squamous cell carcinoma. Epidermal growth factor receptor. *Int J Radiat Oncol Biol Phys* 2003; **55**:713–723.

29 Moulder SL, Yakes FM, Muthuswamy SK, Bianco R, Simpson JF, Arteaga CL. Epidermal growth factor receptor (HER1) tyrosine kinase inhibitor ZD1839 (Iressa) inhibits HER2/neu (erbB2)-overexpressing breast cancer cells in vitro and in vivo. *Cancer Res* 2001; **61**:8887–8895.

30 Normanno N, Campiglio M, De LA, Somenzi G, Maiello M, Ciardiello F, *et al*. Cooperative inhibitory effect of ZD1839 (Iressa) in combination with trastuzumab (Herceptin) on human breast cancer cell growth. *Ann Oncol* 2002; **13**:65–72.

31 Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol* 1995; **19**:183–232.

32 Bianco R, Shin I, Ritter CA, Yakes FM, Basso A, Rosen N, *et al*. Loss of PTEN/MMAC1/TEP in EGF receptor-expressing tumor cells counteracts the antitumor action of EGFR tyrosine kinase inhibitors. *Oncogene* 2003; **22**:2812–2822.

33 Janmaat ML, Giaccone G. The epidermal growth factor receptor pathway and its inhibition as anticancer therapy. *Drugs Today (Barc)* 2003; **39** (**Suppl C**):61–80.

34 Van Schaeybroeck S, Karaiskou-McCaul A, Kelly D, Longley D, Galligan L, Van Cutsem E, *et al*. EGFR activity determines response of colorectal cancer cells to gefitinib (Iressa) alone and in combination with chemotherapy. *Clin Cancer Res* 2005; **11**:7480–7489.

35 Van Schaeybroeck S, Kyula J, Kelly DM, Karaiskou-McCaul A, Stokesberry SA, Van Cutsem E, *et al*. Chemotherapy-induced epidermal growth factor receptor activation determines response to combined gefitinib/chemotherapy treatment in non-small cell lung cancer cells. *Mol Cancer Ther* 2006; **5**:1154–1165.