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Original Article

Chloroquine potentiates the anti-cancer effect of lidamycin on non-small cell lung cancer cells *in vitro*

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Aim: To assess the synergistic actions of lidamycin (LDM) and chloroquine (CQ), a lysosomal enzyme inhibitor, in human non-small cell lung cancer (NSCLC) cells, and to elucidate the potential mechanisms.
Methods: Human NSCLC cell lines A549 and H460 were treated with CQ and/or LDM. Cell proliferation was analyzed using MTT assay, and apoptosis was quantified using flow cytometry. Western blotting was used to detect the protein levels of caspase 3, PARP, Bcl-2, Bax, p53, LC3-I and LC3-II. A H460 cell xenograft model in BALB/c nude mice was used to evaluate the anticancer efficacy of CQ and LDM *in vivo*.
Results: Both LDM and CQ concentration-dependently suppressed the proliferation of A549 and H460 cells *in vitro* (the IC_{50} values of LDM were 1.70±0.75 and 0.043±0.026 nmol/L, respectively, while the IC_{50} values of CQ were 71.3±6.1 and 55.6±12.5 μmol/L, respectively). CQ sensitized both NSCLC cell lines to LDM, and the majority of the coefficients of drug interaction (CDIs) for combination-doses were less than 1. The ratio of apoptosis of H460 cells induced by a combined treatment of CQ and LDM (77.0%±5.2%) was significantly higher than those caused by CQ (23.1%±4.2%) or by LDM (65.1%±4.1%) alone. Furthermore, the combined treatment markedly increased the cleaved PARP and cleaved caspase 3 in H460 cells, which were partly reversed by pretreatment with the caspase inhibitor zVAD.fmk. zVAD.fmk also partially reversed the inhibitory effect of the combination treatment on the proliferation of H460 cells. The combination therapy group had a notable increase in expression of Bax and a very slight decrease in expression of Bcl-2 and p53 protein. LDM alone scarcely affected the level of LC3-II in H460 cells, but slightly reduced CQ-induced LC3-II expression. 3-MA, an autophagy inhibitor also sensitized H460 cells to LDM. In nude mice bearing H460 cell xenograft, administration of LDM (25 μg/kg, iv) and CQ (60 mg/kg, ip) suppressed tumor growth by 57.14% and 73.02%, respectively.
Conclusion: The synergistic anticancer effect of LDM and CQ *in vitro* results from activation of a caspase-dependent and p53-independent apoptosis pathway as well as inhibition of cytoprotective autophagy.

Keywords: lung cancer; anticancer drug; lidamycin; chloroquine; drug interaction; synergism; apoptosis; autophagy

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Introduction

Lung cancer is the most common cause of cancer-induced death worldwide. Surgical resection plays a major role in the treatment of non-small cell lung cancer (NSCLC), but the tumor is often detected in a progressive and inoperable phase. Therefore, chemotherapy is an important means for the treatment of lung cancer. Because of toxicity, the application of antitumor drugs in the clinic is limited despite many years of research on drug development[1]. In recent years, combination therapy for lung cancer has received increasing attention[2, 3].

Lidamycin (LDM, also named C-1027), which was isolated from a soil sample collected from the Qian-jiang area of China[4], is extremely cytotoxic to tumor cells and has been shown to decrease the growth of human tumor xenografts[5]. LDM is currently undergoing a phase II clinical trial[6]. LDM causes double-stranded DNA breakage and tumor cell death by binding to DNA in the minor groove[7]. An excessive dose of systemically administered LDM will generate unacceptable levels of toxicity to normal cells, especially pulmonary cells. Therefore, attempts have been made to enhance the therapeutic effectiveness of LDM therapy while reducing overall toxicity. Previous studies focus on two aspects: the combined use of LDM with another chemotherapeutic agent or the creation of fusion proteins with specific target capabilities.

Chloroquine diphosphate (CQ), an anti-malarial drug, shows potential anti-cancer effects, such as the inhibition of cell growth in human lung cancer A549 cells, human breast cancer cells, and glioma cells[8–10]. In addition, CQ therapy enhances the inhibitory effects of other chemotherapeutic agents on tumors. For example, CQ enhances the inhibitory

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effects of 5-fluorouracil (5-FU), which exerts cytotoxic effects via the alteration of thymidylate synthetase activity or via incorporation into RNA and DNA, for treatment of colorectal cells[11].

CQ, a lysosomal enzyme inhibitor, inhibits late states of autophagy by changing the pH of lysosomes and affects the degradation of proteins wrapped in the autophagosome. The late period of autophagy is blocked, and the number of apoptotic cells increases. When functions of the autophagosome are broken, LC3-II will continue to gather in the membrane of the autophagosome. The chemosensitizing effect of CQ, which is a promising strategy to improve cancer treatment, partly depends on its ability to suppress autophagy[12]. Inhibition of autophagy with 3-MA, CQ, or Beclin-1 shRNA reinforces the death of human salivary gland adenoid cystic carcinoma (ACC) cells after cis-diamminedichloroplatinum (CDDP) treatment[13]. CQ is a promising candidate for combination with chemotherapeutic agents (*eg*, LDM) for improving clinical outcomes through autophagy inhibition and its lysosomotropic properties. Furthermore, because CQ is already in widespread use in humans for many years, it has a great advantage over chemotherapeutic agents that have previously been used in association with LDM, such as 5-FU and CDDP, in that it can be introduced to the clinical settings of cancer therapy without the performance of animal or phase I studies. In addition, its wide therapeutic window makes CQ more usable in the clinic.

The present study was designed to investigate the effect of LDM and CQ combined therapy on NSCLC. Proliferation, apoptosis, and the impact on cellular autophagy following LDM and/or CQ therapy were evaluated using A549 and H460 cells and a H460 xenograft model.

Materials and methods

Reagents

Rabbit anti-caspase 3 antibody, rabbit anti-PARP antibody, rabbit anti-Bcl-2 antibody, rabbit anti-Bax antibody, and p53 antibody were all purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti-LC3B antibody was obtained from Sigma (St Louis, MO, USA). Mouse anti-β-actin antibody was from ZSGB-BIO (Beijing, China). CQ was purchased from Sigma (Deisenhofen, Germany) and prepared initially as a 20 mmol/L stock solution by dissolving in physiological saline (Minkang, China). 3-Methyladenine (3-MA), an autophagy inhibitor, was purchased from Sigma (Deisenhofen, Germany) and dissolved in RPMI-1640 medium to the desired concentration before the experiment. Lidamycin (LDM) was provided by Dr Liang LI from our institute and dissolved in physiological saline as a 1 μmol/L stock. Before use in experiments, each stock solution (such as LDM or CQ) was diluted with RPMI-1640 medium to reach the desired final concentration. N-Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk), the caspase 3 inhibitor, was purchased from Beyotime (Haimen, China). Calcium- and magnesium-free phosphate-buffered saline [PBS (–)] was from Thermo Fisher Scientific, Inc (Hudson, NH, USA). All other chemicals were analytical reagents, made in China, unless otherwise indicated.

Cell culture

The human non-small cell lung cancer (NSCLC) cell lines A549 and H460 were kept in our laboratory. Cells were cultured in RPMI-1640 (Thermo Fisher Scientific, Inc, Hudson, NH, USA) medium supplemented with 10% fetal calf serum (Gibco, Gaithersburg, MD, USA), 1% penicillin/streptomycin (North China Pharmaceutical Co, China) and incubated in a 5% CO_2 incubator at 37 °C.

Cell proliferation analysis

A549 and H460 cells were seeded in 96-well plates. Twelve hours later, different doses of LDM and CQ were added, and the cells were incubated for 24, 48, or 72 h. Each independent experiment was performed three times. After the designated time, 20 μL of MTT (5 mg/mL, Sigma) was added into every well, and the samples were then incubated at 37 °C for 4 h. Supernatant was carefully aspirated and added into 150 μL of DMSO; after oscillating 10 min, absorbance was measured at a wavelength of 570 nm. Cell proliferation was calculated as the ratio of each experimental condition to the control (untreated cells).

Evaluation of the combined effects of drugs

The effect of drug interaction was evaluated by the coefficient of drug interaction (CDI), which was calculated as follows:

$$CDI=AB/(A\times B)$$

AB is the survival rate of the combined effects of both drugs. A and B are the survival effects of each drug alone. When CDI<1, the two drugs have a synergistic effect[14].

Apoptosis analysis by flow cytometry

H460 cells were prepared and treated as described above for 20 h and then fixed and stained with 10 μL of Annexin V-FITC and 5 μL of PI, using the Annexin V-FITC/PI Apoptosis Detection Kit (Biosea Biotechnology, Beijing, China) according to the manufacturer's instructions. Flow cytometric analysis was performed on 1×10^4 cells per sample and analyzed using a FACS Calibur. Each independent experiment was performed three times.

Western blot analysis

Cells were treated with chemicals, digested using trypsin (Gibco) and then harvested. Cells were lysed with 100 μL of cell lysis buffer [50 mmol/L Tris-HCl; pH 8.0; 2% NP-40; 150 mmol/L NaCl; 0.2% SDS; 0.5% sodium deoxycholate; and 1% PMSF (Beyotime, Jiangsu, China)] for 30 min on ice. After centrifugation at 15294×*g* for 15 min at 4 °C, the supernatant was collected. Protein concentration was determined by the BCA Protein Assay Kit (Thermo, USA). The same amount of total protein in each sample (35–45 μg) was added to 5×loading buffer, and the samples were adjusted to an equal volume using 1×loading buffer. Then, the samples were separated by SDS-PAGE on a 10% or 15% polyacrylamide gel and transferred to

a polyvinylidene difluoride membrane (Nuprotein, China). The blotted membranes were blocked with 5% skim milk for 2 h and were incubated with each primary antibody overnight at 4 °C. The membrane was then washed 6 times with TBST (0.05% Tween 20 in Tris-buffered saline) for every 5 min and incubated with the secondary antibodies (diluted 1:5000) as appropriate [goat-anti-mouse IgG and goat-anti-rabbit IgG conjugated to horseradish peroxidase were from ZSGB-BIO (Beijing, China)]. The immunoreactive bands were visualized by enhanced chemiluminescence using the ECL detection system (Millipore, Germany). The images were captured with ChemiImager 5500 (Alpha Innotech) and the integral optical density values were examined for each group using Gel-Pro analyzer. All Western blots shown are representative of at least two independent experiments.

Lung cancer xenografts and treatments

Animal experiments were approved by the Experimental Animal Center of our institute. Six- to eight-week-old female BALB/c mice were ordered from Vital River Company (Beijing, China) and housed under pathogen-free conditions in microisolator cages with laboratory chow and water. H460 cells suspended in serum-free medium (10^7/0.4 mL) were injected into the right armpits of nude mice. When the tumor reached a certain size, it was removed and cut into 2×2×2 mm^3 pieces. The tumor was then transplanted into the right armpits of fresh mice using the trocar. Until tumors grew to approximately 100 mm^3, the mice were randomized into six groups (6/group) according to tumor volumes and body weights. The mice were administered LDM (25 μg/kg, once a week, iv by tail) and CQ (60 $mg \cdot kg^{-1} \cdot d^{-1}$; ip). Tumor long and short diameters (Ref as a and b, respectively) were measured using caliper measurements three times a week and the body weights were recorded. The tumor volume was obtained according to the formula, $V=ab^2/2$. The animal experiment lasted for 28 d.

Statistical analysis

All of the experiments were repeated at least three times. Data were expressed as means±SD. Statistical significance was determined by independent-samples *t*-test. A value of $P<0.05$ was considered a significant difference.

Results

LDM and CQ decreased the viability of A549 and H460 cells

Cell viability decreased after treatment with LDM for 48 h in a dose-dependent manner (Figure 1A). The IC_{50} values (95% confidence limits) were (1.70±0.75) nmol/L for A549 cells and (0.043±0.026) nmol/L for H460 cells. On the other hand, CQ inhibited the proliferation of A549 and H460 cells in a time- and dose-dependent manner. The IC_{50} values of CQ at 48 h were 71.3±6.1 μmol/L for the A549 cells and 55.6±12.5 μmol/L for H460 cells (Figure 1B). The H460 cells were more sensitive to LDM or CQ than the A549 cells. Based on these results, we selected the dose of 25 or 50 μmol/L of CQ in a 48-h-treatment for the further experiments.

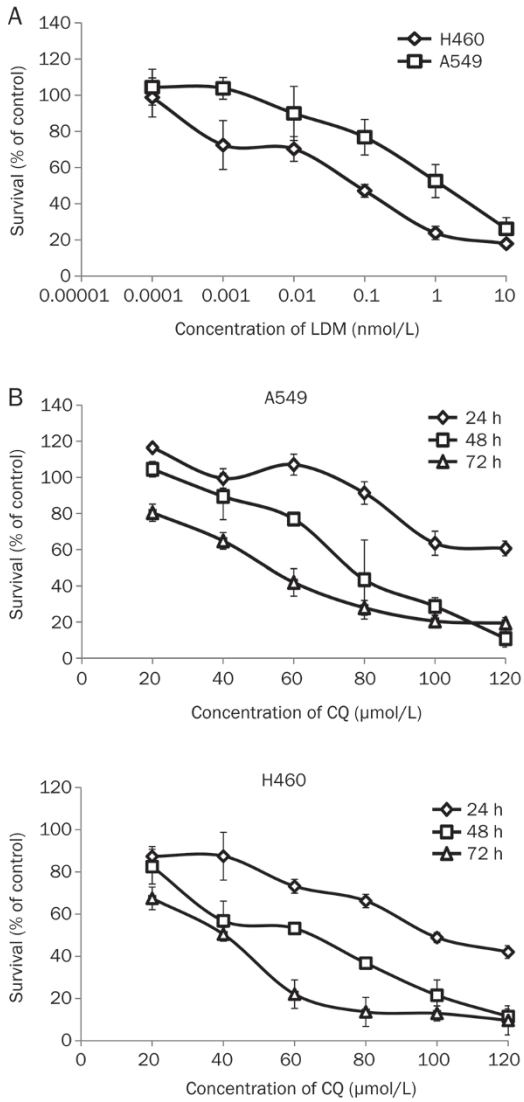

Figure 1. Effect of LDM and CQ on the proliferative activity of A549 and H460 cells. (A) The proliferative activity of A549 and H460 cells treated with LDM (0.0001, 0.001, 0.01, 0.1, 1, or 10 nmol/L) for 48 h assessed by the MTT assay. The y-axis represents the survival rate, calculated as the ratio to the control (untreated cells). (B) The proliferative activity of A549 and H460 cells treated with CQ (20–120 μmol/L) for 24, 48 and 72 h assessed by the MTT assay. The y-axis represents survival rate, calculated as the ratio to the control (untreated cells). Values were given as mean±SD. *n*=3. Each independent experiment was performed in triplicate.

CQ and LDM individually reduced the growth of H460 xenografts in BALB/c nude mice

Based on the above results, CQ and LDM individually inhibited the proliferation of non-small cell lung cancer cells *in vitro*; thus, we explored whether CQ or LDM would reduce the growth of tumor *in vivo*. Drug treatment began according to the method described above at the 10th d after the tumor blocks were implanted in BALB/c nude mice. There were no deaths in any of the experimental groups during the experi-

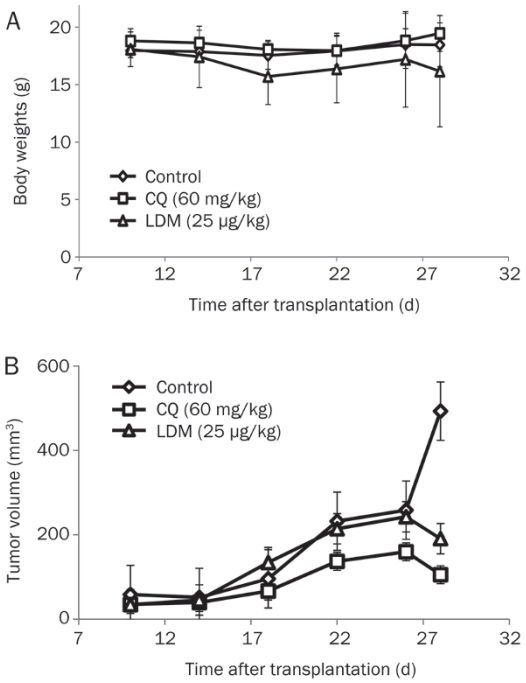

Figure 2. Inhibitory effect of LDM and CQ on the growth of lung cancer H460 xenografts in nude mice. Tumor bearing mice were treated with CQ (60 mg·kg^{-1}·d^{-1}; ip), or LDM (25 μg/kg, on d 7 and 15, iv by tail) after tumor inoculation as described in the "Methods". (A) Changes of body weight of nude mice bearing human lung cancer H460 xenografts. (B) Effects of LDM and CQ on the growth of H460 xenografts in nude mice, expressed by changes of the tumor volume. Tumor volumes were measured every three days. Values were given as mean±SD. *n*=6.

ment. The average body weight in the LDM group decreased from the 4th day after drug treatment, at the termination of the experiment, it had decreased by 10.62% compared with the pretreatment (Figure 2A). Therefore, the dose of LDM was tolerated. The weights of the control group and the CQ group were stable. On the other hand, 60 mg/kg CQ and 25 μg/kg LDM suppressed tumor growth at rates of 73.02% and 57.14%, respectively, compared to the control at the end of the experiment (Figure 2B). In brief, the two drugs possessed inhibitory effects on tumor growth *in vitro* and *in vivo*.

CQ enhanced the LDM inhibitory effect on the cell viability

Combinatorial treatment is an important method in cancer chemotherapy. CQ, in combination with a variety of anticancer drugs, such as erlotinib, gefitinib, and tamoxifen, has been actively evaluated in clinical trials. Here, we investigated the synergistic effect of LDM and CQ on the proliferation of NSCLC cells. The cells were pretreated with 25 and 50 μmol/L of CQ and later dosed with LDM. CQ sensitized H460 cells and A549 cells to LDM, and the majority of CDIs for the combination-doses were less than 1 (Figure 3A, 3B). Therefore, a synergistic effect between CQ and LDM was observed. As expected, H460 cells were more sensitive to LDM than A549 cells from Figure 3A. Based on the above results the following were chosen for the further experiments: H460 cells alone, dose of CQ (50 μmol/L) and LDM (0.5 nmol/L), H460 cells were then treated with CQ (50 μmol/L), LDM (0.5 nmol/L) and the combination therapy (CQ pretreatment for 2 h) for 20 h. Based on the images obtained by microscopy, the combined treatment group had fewer cells, more dead individual cells, and more cellular debris (indicated by arrows) compared with CQ or LDM alone (Figure 3C).

CQ potentiated apoptosis of H460 cells induced by LDM

To determine the effects of the two drugs on apoptosis, we treated H460 cells with CQ (50 μmol/L), LDM (0.5 nmol/L) and both (CQ 2 h pretreatment) for 20 h. Annexin V-FITC and PI staining represented early and late apoptotic and necrotic cells. The apoptotic cells were elevated in the CQ+LDM group compared with the CQ or LDM group (CQ: 23.05%±4.15%, LDM: 65.12%±4.10%, CQ+LDM: 77.00%±5.20%) (Figure 4A).

After we determined that CQ in combination with LDM could induce H460 cells to undergo apoptosis, Western blot analysis was applied to investigate changes in marker proteins in the apoptotic pathway. We found that the CQ+LDM group had increased expression of cleaved-caspase 3. PARP was the substrate of activated caspase 3, and the result showed that PARP decreased and its cleavage increased after the combined treatment (Figure 4B).

CQ and LDM cooperated to induce apoptosis via a caspase-dependent and p53-independent pathway.

To determine whether caspase activity is required for cell death induced by the combination of CQ and LDM, we tested the effect of the broad-range caspase inhibitor zVAD.fmk. Notably, zVAD.fmk significantly reduced the CQ- and LDM-triggered increases in levels of cleaved-PARP and cleaved-caspase 3 (Figure 5B). zVAD.fmk also partially reversed the inhibitory effect of the combination treatment on the proliferation of H460 cells (Figure 5A). These results showed that cell death following treatment with CQ and LDM occurred in a caspase-dependent manner.

p53 often plays a direct pro-apoptotic role by mediating the transcriptional activation of proteins such as Bax and the inactivation of anti-apoptotic Bcl-x_L (such as Bcl-2)[15]. In addition, changes in Bcl-2 and Bax often characterize apoptotic changes in cells. Western blot analysis showed that the combination therapy group had a notable increase in expression of Bax and a very slight decrease in expression of Bcl-2 (Figure 5C). However, the expression of p53 protein was slightly decreased when CQ was combined with LDM (Figure 5C). The results confirmed that CQ combined with LDM induced p53-independent apoptosis and that increased levels of the pro-apoptotic protein Bax subsequently initiated apoptosis following combination treatment.

Effect of LDM and CQ on autophagy of H460 cells

CQ, an autophagy inhibitor, exerted a significant synergistic effect with LDM. To determine whether the synergy was associated with autophagy, we treated H460 cells with 3-MA, a specific inhibitor of autophagy, and LDM. The cells were

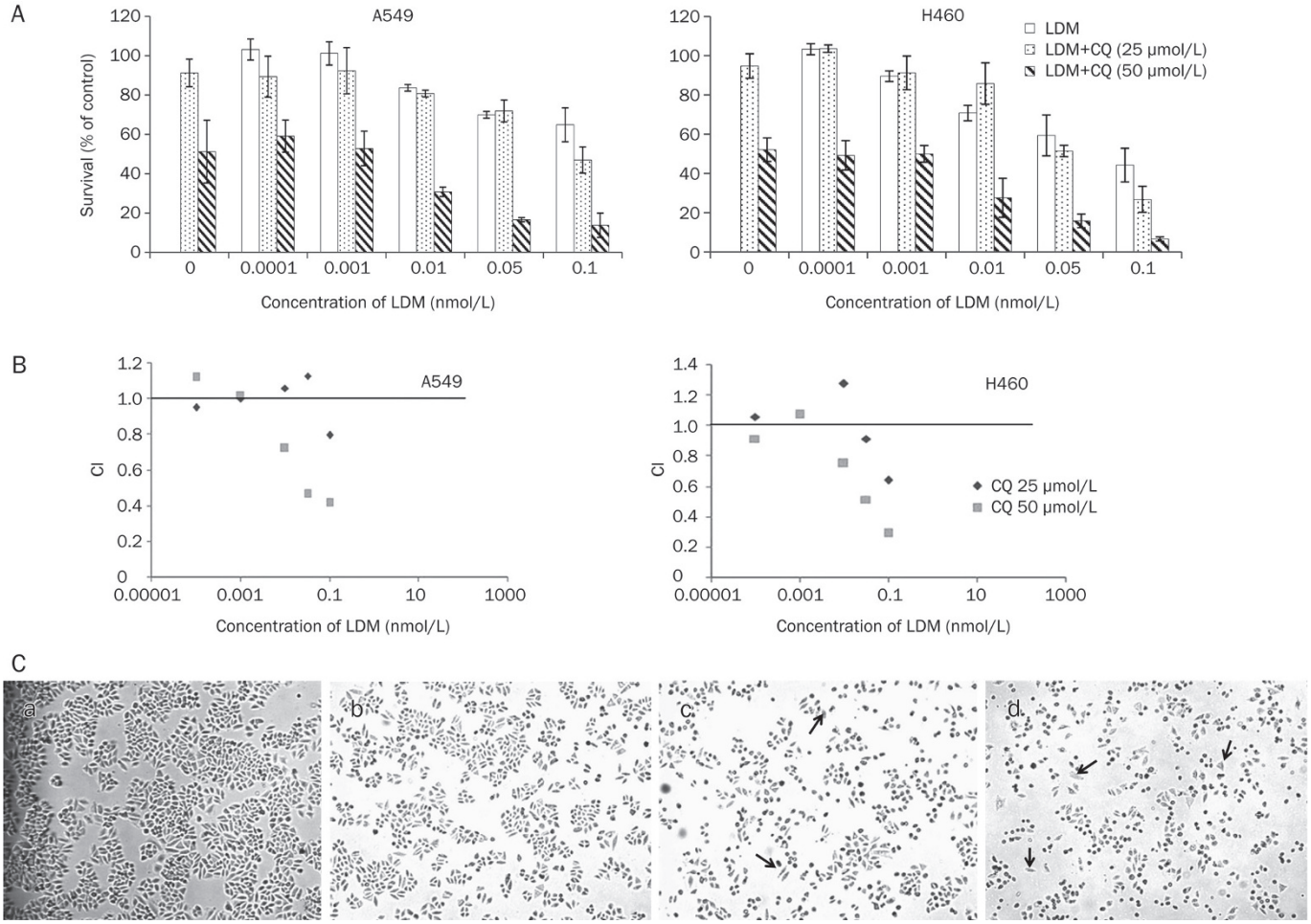

Figure 3. Cell viability of A549 and H460 cells in response to the combination of CQ and LDM. (A) The effect of CQ combined with LDM on the proliferative activity of A549 and H460 cells was investigated by MTT assay. The cells were pretreated with CQ (25 and 50 μmol/L) before exposed to LDM (0.0001, 0.001, 0.01, 0.05, and 0.1 nmol/L) for another 48 h. The data were calculated as the ratio to control (untreated cells). Values were given as mean±SD. *n*=3. (B) CDI value is a quantitative measure of the degree of interaction between different drugs. When CDI values between 1 and 0.7 indicate slight to moderate synergism; CDI values of 0.7 to 0.3, strong synergism. Each independent experiment was performed in triplicate. (C) H460 cells were treated with CQ (50 μmol/L), LDM (0.5 nmol/L) and both (CQ pretreatment for 2 h) for 20 h. Observed under the microscope [(a) Control; (b) CQ; (c) LDM; (d) CQ+LDM], the number of cells in group CQ+LDM was reduced significantly compared with group LDM or CQ alone, and group CQ+LDM had more cellular debris (as shown by arrows).

pre-treated with 3-MA (4 mmol/L) for 2 h, after which doses of LDM were added. Notably, 3-MA sensitized H460 cells to LDM (Figure 6A). The two autophagy inhibitors, in combination with LDM, both have a synergistic effect. We can infer that autophagy may be a protective mechanism and a critical pathway in the synergistic effect of CQ and LDM.

To investigate the specific change of autophagy, the expression of LC3 in H460 cells was examined by Western blot after the cells were treated with CQ (50 μmol/L), LDM (0.5 nmol/L) and a combination of the two (CQ pretreatment for 2 h) for 2 and 24 h. LC3-II expression was scarcely affected by treatment with LDM alone but slightly decreased after combination therapy (LDM and CQ) compared with CQ alone; this measurement was described as the density (Figure 6B).

Discussion

LDM is a potential anticancer drug in Phase II clinical trials, but attempts have been made in previous studies to optimize the structure of LDM, improve the effects of the therapy, and avoid its side effects by combining it with other chemotherapeutic agents. LDM combined with gefitinib, 5-FU, paclitaxel (TAX), doxorubicin (DOX), novelbine (NVB), or CDDP shows a significant synergistic anti-tumor effect *in vitro* and *in vivo*[16, 17]. Compared to chemotherapeutic drugs, CQ has been used to treat a variety of diseases, including malaria, rheumatoid arthritis, systemic lupus erythematosus, and amebic hepatitis, and it has a wide therapeutic window[18, 19].

Recently, the ability of CQ to block autophagy through the inhibition of lysosomal proteases and autophagosome-

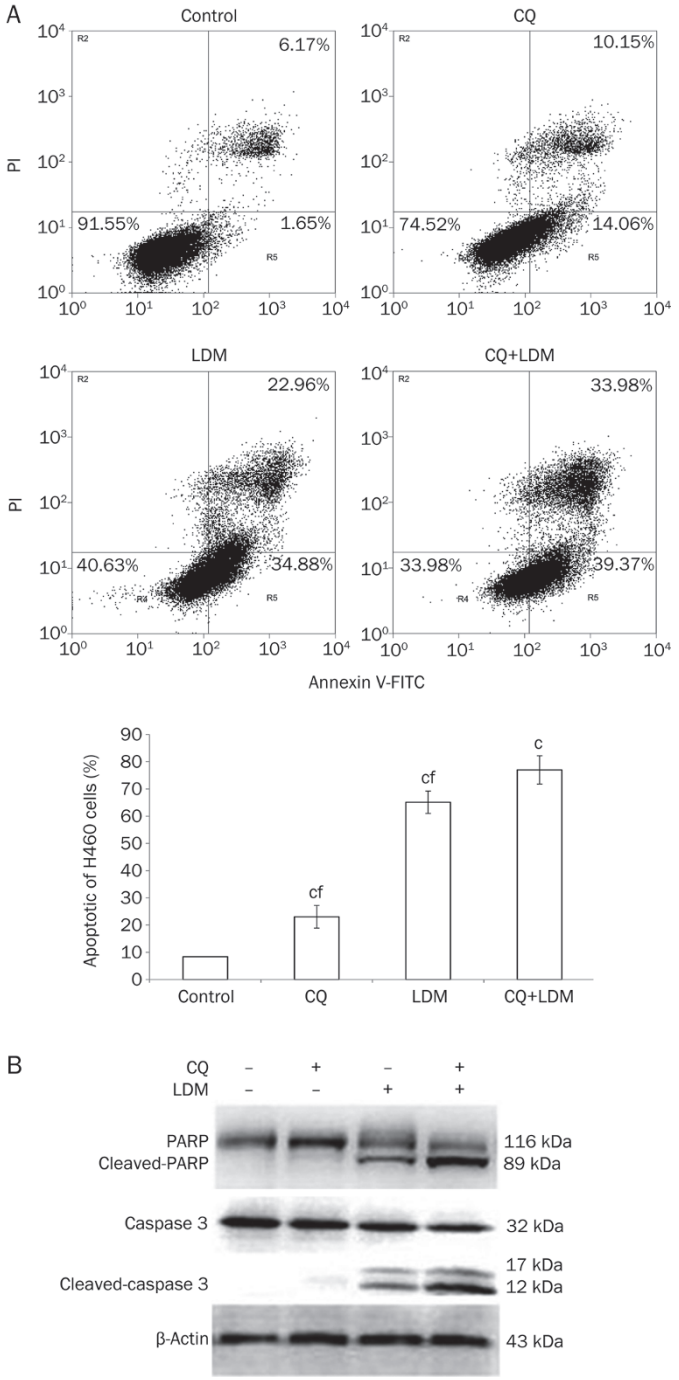

Figure 4. CQ enhances LDM's effects in inducing apoptosis in H460 cells. H460 cells were treated with CQ (50 μmol/L), LDM (0.5 nmol/L) and both (CQ pretreatment for 2 h) for corresponding time. (A) The proportion of apoptotic cells were evaluated by FCM using annexin V-FITC/PI staining in H460 cells after the treatment above for 48 h. The percentage of apoptotic cells increased in combined treatment group compared with LDM alone (65.12% *vs* 77.00% for LDM alone and CQ+LDM, respectively). Three independent experiments were performed. The data were represented as Mean±SD. n=3. ^{c}P<0.01 *vs* control. ^{f}P<0.01 *vs* CQ+LDM. (B) The influence of LDM and CQ (alone or both) on expression of caspase 3 and PARP.

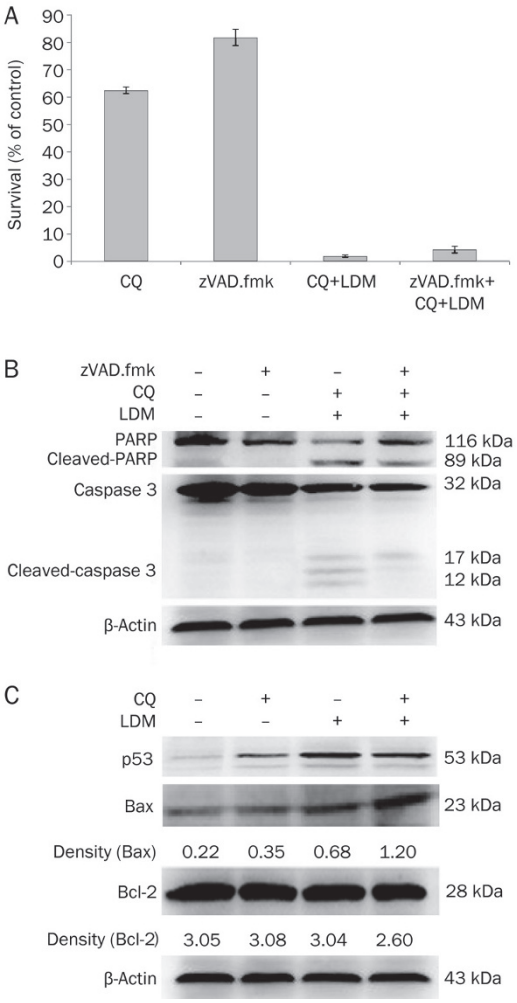

Figure 5. Effect of the combination of LDM and CQ on apoptosis pathways. H460 cells were treated with both CQ and LDM (CQ pretreatment for 2 h) or none for 20 h. zVAD.fmk (100 μmol/L) were added before CQ and LDM for 1.5 h. (A) zVAD.fmk partly reversed the inhibitory effect of CQ combined with LDM on the proliferation of H460 cells. Mean±SD. n=3. (B) zVAD.fmk reduced the increasing expression of cleaved-PARP and cleaved-caspase 3 induced by CQ and LDM. (C) H460 cells were treated with CQ (50 μmol/L), LDM (0.5 nmol/L) and both (CQ pretreatment for 2 h) for 20 h. The influence of LDM and CQ (alone or both) on expression of p53, Bax, and Bcl-2. The expression levels of Bax and Bcl-2 were measured as the density by the Gel-Pro software, standardized by the density of β-actin.

lysosomal fusion events has attracted further interest in cancer treatment[20]. Because autophagy is thought to be an important cell-survival pathway in the development of cancer, CQ has been combined with diverse chemotherapeutic drugs or radiation to enhance its killing effect on cancer cells. It is reported that CQ can overcome primary resistance to trastuzumab in patients with HER2-positive breast cancer by preventing the accumulation of autophagolysosomes formed in the presence of trastuzumab[21]. CQ has also been confirmed to potentiate the antitumor activity of erlotinib, the inhibitor of epidermal

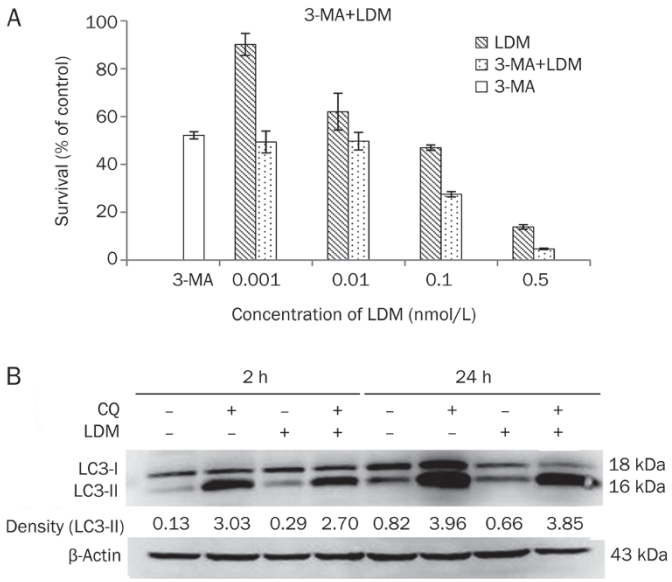

Figure 6. Effect of the combination of LDM and CQ on autophagy. (A) The effect of 3-MA combined with LDM on the proliferative activity of H460 cells was investigated by MTT assay. The cells were pretreated with 3-MA (4 mmol/L) before exposed to LDM (0.001, 0.01, 0.1, and 0.5 nmol/L) for another 48 h. The data were calculated as the ratio to control (untreated cells). Values were given as mean±SD. *n*=3. (B) The expression of LC3-II was quantified by Western blot in H460 cells treated with CQ (50 μmol/L), LDM (0.5 nmol/L) and both (CQ pretreatment for 2 h) for 2 h (left) and 24 h (right). The expression levels of LC3-II were measured as the density by the Gel-Pro software, standardized by the density of β-actin.

growth factor receptor (EGFR)[22], and of ABT-737, the Bcl-2 inhibitor[23]. Our results showed that LDM and CQ individually reduced tumor growth *in vitro* and *in vivo*. Therefore, we tested the effect on the proliferation of cells of a new combination therapy (CQ and LDM), investigated the mechanisms underlying this effect, and studied the changes in autophagy after LDM treatment for the first time. Moreover, we briefly discuss the relationship between autophagy and the apoptosis induced by the combination of LDM and CQ.

Apoptosis, or programmed cell death I, is critical for tissue homeostasis in multicellular organisms and can often be divided into two pathways at the molecular level: the mitochondrial apoptotic pathway and the death receptor pathway. The Bcl-2 family is composed of apoptosis-related proteins located in the mitochondrial membrane, including Bcl-2 subfamily members (*eg*, Bcl-2 and Bcl-x_L) that inhibit apoptosis, and Bax subfamily members (*eg*, Bax and Bak) that promote apoptosis[24]. Both pathways activate a series of cysteine proteases (caspases), eventually leading to apoptosis, so caspase is a hub of the two pathways. However, apoptosis is involved in the absence of caspase circumstances. The rapid reactive oxygen species (ROS) generation induced by curcumin causes the release of apoptosis-inducing factor (AIF), which travels from mitochondria to the cytosol and nucleus, leading to apoptosis without activating caspase 3[25]. Based on the above theory, we further investigated apoptosis. In the present study, CQ had little effect on caspase 3 or PARP, but when combined with LDM, it increased the expression levels of cleaved-caspase 3 and cleaved-PARP. These increased expressions, as well as cell proliferation, were largely suppressed by the broad-range caspase inhibitor zVAD.fmk. In addition, the combination of CQ and LDM increased the expression level of Bax notably. We concluded that cell death upon co-treatment of CQ and LDM was due to a Bax-related and caspase-dependent apoptosis pathway.

Autophagy, a conserved catabolic process known as a type II cell death program, has dual roles in mammalian cells[26]. It can degrade long-cycle proteins and cytoplasmic organelles under the circumstances of hunger or hypoxia and it can produce small molecules and ATP to maintain cell survival in starvation, after being induced by drugs[27]. Meanwhile, autophagy can direct or collaborate with apoptosis to produce cell death[28]. The Western blot analysis for the expression of LC3 I and LC3 II, two protein markers of autophagy, showed that CQ combined with LDM inhibited autophagy compared with LDM alone. In addition, 3-MA, another autophagy inhibitor, also had a synergistic effect with LDM. Autophagy appeared to play a self-defense mechanistic role in LDM-treated lung cancer cells.

Complex linkages exist between the two types of programmed cell death: apoptosis and autophagy[29]. The essential autophagy-inducing protein, Beclin 1, is bound to and inhibited by Bcl-2 or the Bcl-2 homolog Bcl-x_L. Moreover, the anti-apoptotic Bcl-2 family may play a role in the inhibition of autophagy, so the crosstalk between the core mechanism that regulating apoptosis and autophagy may focus on Beclin 1 or Bcl-2[30]. In our experiments, the combination of LDM and CQ inhibited autophagy while inducing apoptosis. Therefore, we hypothesized that autophagy was a catabolic process that provided nutrients through macromolecular degradation, thus replenishing the vanishing energy reserves of the starving cell and preventing a bio-energetic catastrophe that would otherwise culminate in cell death. However, anti-apoptotic protein Bcl-2, which inhibits autophagy, shows no significant changes with the combination treatment. Thus, we recommend additional studies on autophagy that investigate more deeply, such as targeting signaling molecules, the crosstalk between autophagy and apoptosis, and so on.

The *in vitro* data suggested that CQ and LDM together are responsible for a significant inhibitory effect on the vitality of NSCLC. In our experiments, despite some meaningful mechanisms and intriguing molecular interactions, the combination therapy (CQ and LDM) exhibited no synergistic or enhanced effects on lung cancer *in vivo* compared with CQ or LDM alone (data not shown). The reasons may be related to the complex environment and metabolism of drugs *in vivo*. Therefore, the selections of dosage and frequency should be further studied.

In summary, there is a synergistic effect on tumor growth *in vitro* with the combination therapy of CQ and LDM. CQ enhances LDM-induced apoptosis of NSCLC via a Bax-related, caspase-dependent, P53-independent pathway and inhibits autophagy, a mechanism that protects cells from death.

Moreover, the inhibition of autophagy might be an attractive strategy to enhance the LDM-induced anti-tumor effect. It is clearly demonstrated that combined therapy with CQ and LDM is an effective and promising strategy for the treatment of lung cancer.

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Author contribution

Shu-zhen CHEN designed the study and edited the paper; Fang LIU performed the research, analyzed the data and wrote the paper; and Yue SHANG performed the mouse experiments.

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