

Dihydroartemisinin sensitizes Lewis lung carcinoma cells to carboplatin therapy via p38 mitogen-activated protein kinase activation

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Abstract. Dihydroartemisinin (DHA), a semi-synthetic derivative of artemisinin isolated from the traditional Chinese herb *Artemisia annua*, is an effective novel antimalarial agent. Studies have suggested that it also exhibits anticancer effects when administered alone or in combination with conventional chemotherapeutic agents. The present study investigated the therapeutic effect of DHA combined with carboplatin (CBP) on Lewis lung carcinoma (LLC) cells and the possible underlying molecular mechanisms. MTT and clonogenic assays demonstrated that the proliferation activity of LLC cells was inhibited in a dose-dependent manner by DHA combined with CBP. In addition, flow cytometry analysis revealed that cell cycle arrest was induced at the G₀/G₁ phase and apoptosis was induced following treatment with the combination. When administered in combination with CBP, DHA exhibited more effective anticancer activity compared with DHA or CBP used alone, via increased apoptosis. Following treatment with DHA with or without CBP, the expression of phosphorylated-p38 mitogen-activated protein kinase (MAPK), which can be inhibited with the selective inhibitor SB202190, was detected by western blotting. To summarize, the results of the present study indicated that DHA may sensitize LLC cells to CBP therapy via the activation of p38MAPK, which suggests that a combined treatment of DHA and CBP may be a potential novel therapeutic schedule for lung adenocarcinoma.

Introduction

Lung cancer has the highest levels of morbidity and mortality of all malignant tumor types, in China and worldwide, and adenocarcinoma is most common type of lung cancer (1). The treatment of lung adenocarcinoma frequently fails, as the majority of patients present with metastatic disease at the point of diagnosis (2). Despite continuous progress in chemotherapeutic drug development and the application of molecular targeted therapy in recent years, the 5-year survival rate of lung cancer has not significantly increased (3,4). Therefore, it is necessary to investigate novel anticancer agents that are highly effective and exhibit low toxicity for clinical intervention in lung adenocarcinoma.

Dihydroartemisinin (DHA), a more water-soluble active metabolite of artemisinin isolated from the Chinese herb *Artemisia annua L* (also called qinghaosu or sweet wormwood), is part of a new generation of drugs against fever and chloroquine- and mefloquine-resistant strains of *Plasmodium falciparum* (5). DHA has been revealed to be capable of inhibiting cell proliferation and inducing apoptosis in cancer cells, including ovarian, leukemia, osteosarcoma and lung cancer cells, when used alone or in combination with conventional chemotherapeutic agents (6-9). In various cell lines, DHA activates signaling pathways, including the Wnt/ β -catenin, nuclear factor- κ B (NF- κ B), mammalian target of rapamycin (mTOR) and caspase signaling pathways (8,10); however, the molecular mechanism underlying the DHA-induced inhibition of cell proliferation and induction of apoptosis requires further investigation.

The mitogen-activated protein kinase (MAPK) cascade is an important signal transducer from extracellular to nuclear and participates in diverse cellular functions, including cell proliferation, differentiation and death (11). At least 4 key proteins of signaling pathways are associated with the MAPK family in eukaryotic cells: Extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), big MAP kinase and p38MAPK (12). p38MAPK was identified as a kinase induced by stress signals (13). Previous studies indicated that p38MAPK may be a positive or negative regulator

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of apoptosis (14,15). Whether p38MAPK signaling is involved in DHA-induced cell proliferation inhibition and apoptosis in lung adenocarcinoma requires further investigation.

In the present study, Lewis lung carcinoma (LLC) cells were incubated with DHA with or without carboplatin (CBP). The viability of the treated cells was observed by MTT and clonogenic assays, cell cycle and apoptotic rate were analyzed by flow cytometry. The expression levels of phosphorylated (p)-p38MAPK or p-JNK in the cell lines were analyzed with western blotting. The present study demonstrated that DHA sensitized LLC cells to CBP therapy via p38MAPK activation, which suggested that a combined treatment with DHA and CBP maybe a potential novel treatment regimen for lung adenocarcinoma therapy.

Materials and methods

Agents. DHA was purchased from Wulingshan Pharm (Chongqing, China) and dissolved in dimethyl sulfoxide (DMSO; Amresco, LLC, Solon, OH, USA) to make a 500 $\mu\text{g/ml}$ stock solution, with a final concentration of 0.5% (v/v) DMSO in the following experiments, as previously described (16). CBP was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and 50 $\mu\text{mol/l}$ was used as the experimental concentration. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies were obtained from Pierce (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The polyclonal rabbit anti-rat antibodies p38MAPK (cat no. 9212) and p-p38MAPK (cat no. 9211) were supplied by Cell Signaling Technology, Inc., Danvers, MA, USA. The mouse monoclonal anti-human antibodies JNK (cat no. sc-7345), p-JNK (cat no. sc-293137), mouse monoclonal anti-human antibody β -actin (cat no. sc-130300), Protein-G-agarose beads and Alexa Fluor 488-conjugated anti-HA antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Cell culture. The Lewis lung carcinoma (LLC) cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI-1640 (Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) and antibiotics (100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin). The cell lines were maintained at 37°C in a humidified air atmosphere with 5% CO₂.

Inhibition assays. To investigate whether MAPK served a role in DHA-induced apoptosis, LLC cells were treated with DHA in the presence of specific inhibitor of a p38MAPK (SB202190, 10 $\mu\text{mol/l}$) or JNK inhibitor (SP600125, 10 $\mu\text{mol/l}$) (Merck KGaA) for 2 h, respectively, prior to further experiments.

MTT assay. To detect the viability of cells following treatment, an MTT assay was performed. Briefly, cells were seeded at a density of 1×10^4 cells/well in 96-well plates. Subsequently, they were incubated with DHA (final concentrations, 5, 10, 20 and 40 $\mu\text{g/ml}$) or CBP (final concentration, 25 $\mu\text{g/ml}$) for 24 h. MTT (20 μl ; Amresco, LLC) was added to each well and the cells were incubated at 37°C for 4 h. Finally, 150 μl DMSO was added. The optical density (OD) value was then evaluated at 570 nm using a microplate reader (Thermo Fisher

Scientific, Inc.). The inhibition rate of cell proliferation was calculated as follows: (OD value of the control group-OD value of the test group) $\times 100\%$ /OD value of the control group. The half-maximal inhibitory concentration (IC₅₀) for DHA and CBP in LLC cells, defined as the dose of DHA and CBP that inhibits cell growth by 50%, was derived from the dose-response curve.

Clonogenic assay. LLC cells in the exponential phase of growth were seeded in 6-well plates (1,000 cells per well) and allowed to adhere. Following treatment with serial concentrations of DHA (5, 10, 20 and 40 $\mu\text{g/ml}$) and/or CBP (25 $\mu\text{g/ml}$) for 24 h, the cells were cultured in fresh medium without drugs until visible colonies formed. The cell colonies were then fixed with 4% paraformaldehyde at room temperature for 30 min and stained with 0.5% crystal violet staining solution at room temperature for 2 h. A total of 5 fields of view per well were counted under an optical microscope at $\times 100$ magnification.

Cell cycle and apoptosis analysis. Cell cycle and apoptosis were analyzed by flow cytometry. Briefly, following incubation with DHA (20 $\mu\text{g/ml}$) or CBP (25 $\mu\text{g/ml}$) for 24 h, 1×10^6 LLC cells were harvested with trypsin and washed twice with ice-cold PBS. The cells were fixed with 70% ethanol at 4°C overnight, then treated with propidium iodide and RNase A at 37°C for 40 min. The samples were then analyzed by flow cytometry (FACScalibur; BD Biosciences, San Jose, CA, USA) for the proportion of cells in G₀/G₁, S or G₂/M cell cycle phase. The sub-G₁ population was scored from hypodiploid DNA content. Data were analyzed using ModFit LT V3.2 software (Verity Software House, Topsham, ME, USA). Apoptosis was determined using the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis kit (BioVision, Inc., Milpitas, CA, USA), according to the manufacturer's instructions. The early apoptotic (Annexin V-FITC-positive) and necrotic/late apoptotic (Annexin V-FITC-positive, PI-positive) cells were quantified as apoptotic cells.

Western blotting. For the detection of various proteins (p38MAPK, p-p38MAPK, JNK or p-JNK), cells were lysed in lysis buffer (Thermo Fisher Scientific, Inc.) and clarified by centrifugation at 12,000 $\times g$ at 4°C for 10 min. The amount of protein was quantified with the Bradford method. A 50- μg mass of cell proteins were loaded onto 10-15% SDS-PAGE gels for electrophoresis and the separated proteins were subsequently electro-transferred onto polyvinylidene membranes. The membranes were blocked with 5% skimmed milk in PBS with Tween-20 at room temperature for 1 h in a container and probed with various primary antibodies (rabbit polyclonal anti-p38MAPK antibodies, cat no. 9212; rabbit polyclonal anti-p-p38MAPK, cat no. 9211 at dilutions of 1:250; mouse-polyclonal anti-JNK, cat no. sc-7345 at a dilution of 1:250; mouse monoclonal anti-p-JNK, cat no. sc-293137 at a dilution of 1:500) overnight at 4°C, and the membranes were washed with TBST and incubated with a secondary rabbit anti-mouse IgG horseradish peroxidase (HRP) antibody (cat no. sc-358917, 1:2,000; Santa Cruz Biotechnology, Inc.) or mouse anti-rabbit IgG-HRP antibody (cat no. sc-2357, 1:2,000 dilution; Santa Cruz Biotechnology, Inc.) diluted in 5% (w/v) dry non-fat milk in TBST for 1 h at room temperature. Finally,

the membranes were washed and the bands were examined using an enhanced chemiluminescence western blotting detection system (National Institutes of Health, Bethesda, MD, USA) and the Bio-Rad ChemiDoc XRS imager (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. All data are presented as the mean \pm standard deviation of ≥ 3 independent experiments. Statistical differences between means were analyzed by an independent-samples t-test. Rates were compared using the χ^2 test. $P < 0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Proliferation is inhibited in LLC cells following treatment with DHA and CBP. An MTT assay was performed to determine the viability of LLC cells. Following treatment with DHA or CBP, the proliferation of LLC cells was inhibited with a dose-dependent manner. The inhibitory rates of DHA or CBP at various concentrations on LLC cells are described in Table I. The IC_{50} values for DHA and CBP in LLC cells in our unpublished materials were 21.94 (18-25 $\mu\text{g/ml}$) and 26.98 $\mu\text{g/ml}$ (22-30 $\mu\text{g/ml}$) respectively, thus 20 $\mu\text{g/ml}$ DHA and 25 $\mu\text{g/ml}$ CBP were selected for use in the subsequent experiments.

To determine whether DHA enhanced the cytotoxicity of CBP, LLC cells were co-treated with 25 $\mu\text{g/ml}$ CBP and 5, 10, 20 or 40 $\mu\text{g/ml}$ DHA for 24 h. Following the treatment, the inhibitory rate of LLC cells increased to 64.81%, which was higher compared with DHA or CBP used alone ($P < 0.05$; Table I). In addition, the clonogenic assay revealed that DHA, CBP and DHA+CBP treatments significantly decreased the colony numbers of LLC cells compared with the control group ($P < 0.05$; Fig. 1). There was no significant difference between the group of DHA (20 $\mu\text{g/ml}$) or CBP (25 $\mu\text{g/ml}$) used alone compared with the control group ($P > 0.05$); however, the colony formation of LLC cells was significantly inhibited by various concentrations of DHA (5, 10, 20 and 40 $\mu\text{g/ml}$) combined with CBP (25 $\mu\text{g/ml}$; $P < 0.05$).

Apoptosis is induced in LLC cells by DHA combined with CBP. In order to investigate the mechanisms underlying the reduction in cell proliferation induced by DHA (20 $\mu\text{g/ml}$) combined with CBP (25 $\mu\text{g/ml}$), the cell cycle distribution and apoptosis rates of LLC cells were assessed by analysis of flow cytometry. As presented in Table II, compared with the control group, the number of cells in the G_0/G_1 cell cycle phase increased, whereas cells in the S and G_2/M cell cycle phases markedly decreased ($P < 0.05$; Fig. 2A), and apoptotic peaks emerged in the DHA, CBP and the combination groups. The apoptotic rates in the DHA, CBP and the combination groups were 7.45, 32.41 and 36.14%, respectively (Fig. 2B). The apoptotic rate of the combination group was distinctly different compared with the control and DHA groups ($P < 0.05$; Table II).

DHA sensitizes LLC cells to CBP-induced apoptosis via p38MAPK activation. To investigate a potential role for MAPKs in the therapeutic effect of DHA combined with CBP

Table I. Effect of DHA or CBP on Lewis lung carcinoma cell proliferation.

Group	Absorbance value	Inhibitory rate (%)
Control	0.54 \pm 0.06	0
CBP (25 $\mu\text{g/ml}$)	0.45 \pm 0.04	16.67
DHA (20 $\mu\text{g/ml}$)	0.48 \pm 0.03	11.11
DHA (5 $\mu\text{g/ml}$) + CBP (25 $\mu\text{g/ml}$)	0.43 \pm 0.05	20.37
DHA (10 $\mu\text{g/ml}$) + CBP (25 $\mu\text{g/ml}$)	0.34 \pm 0.03	37.04
DHA (20 $\mu\text{g/ml}$) + CBP (25 $\mu\text{g/ml}$)	0.29 \pm 0.04	46.30
DHA (40 $\mu\text{g/ml}$) + CBP (25 $\mu\text{g/ml}$)	0.19 \pm 0.05	64.81

DHA, dihydroartemisinin; CBP, carboplatin.

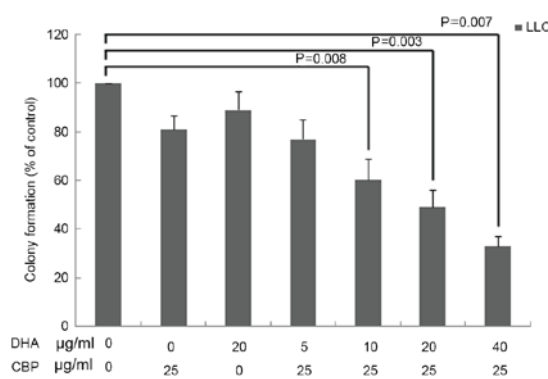


Figure 1. The anti-proliferative effects of DHA, CBP and DHA+CBP on LLC cells. LLC cells were treated with DHA, CBP and DHA+CBP for 24 h. Subsequently, the cells were grown in fresh medium without drugs to form colonies. The colony formation ratio (% of control) was determined. DHA, dihydroartemisinin; CBP, carboplatin; LLC, Lewis lung carcinoma.

in LLC cells, the levels of p-p38MAPK and p-JNK in each treatment group were examined by western blot analysis. LLC cells exhibited constitutive p-p38MAPK activity following mono-DHA or mono-CBP treatment. Furthermore, compared with the control, DHA or CBP groups, p-p38MAPK was upregulated in the combined treatment group. Conversely, induction of JNK phosphorylation was not observed in all four groups. In addition, no p-p38MAPK signal was detected in the SB202190 group (Fig. 3). Among the four groups, there was no difference in p-JNK expression level. These results suggested that in LLC cells, DHA with CBP co-treatment *in vitro* can abrogate mono-DHA or mono-CBP-induced p38MAPK activity, but not JNK phosphorylation, which may be associated with the enhanced rate of apoptosis observed.

Discussion

The results of the present study revealed that DHA inhibits cell proliferation and induces apoptosis in LLC cells in a concentration-dependent manner. Artemisinin, isolated from the traditional Chinese herb *Artemisia annua* is an effective novel antimalarial drug with low toxicity. The derivatives of artemisinin include DHA, artemether, artemotil and

Table II. Analysis for the effect of DHA or/and CBP on cell cycle and apoptosis in Lewis lung carcinoma cells by flow cytometry.

Group	G ₀ /G ₁ phase	S phase	G ₂ /M phase	Apoptotic rate (%)
Control	52.90±5.25	39.25±4.32	5.85±1.28	2.95±0.84
DHA (20 μg/ml)	56.65±6.34	38.46±2.10 ^a	4.89±1.15 ^b	7.45±3.19 ^a
CBP (25 μg/ml)	61.94±3.37 ^a	34.08±3.54	4.21±1.12	32.41±3.73 ^b
DHA (20 μg/ml) + CBP (25 μg/ml)	65.07±7.40 ^a	31.79±6.27 ^a	3.64±0.88 ^a	36.14±4.98 ^b

Values are mean ± standard deviation. ^aP<0.05 and ^bP<0.01 compared with the control group. DHA, dihydroartemisinin; CBP, carboplatin.

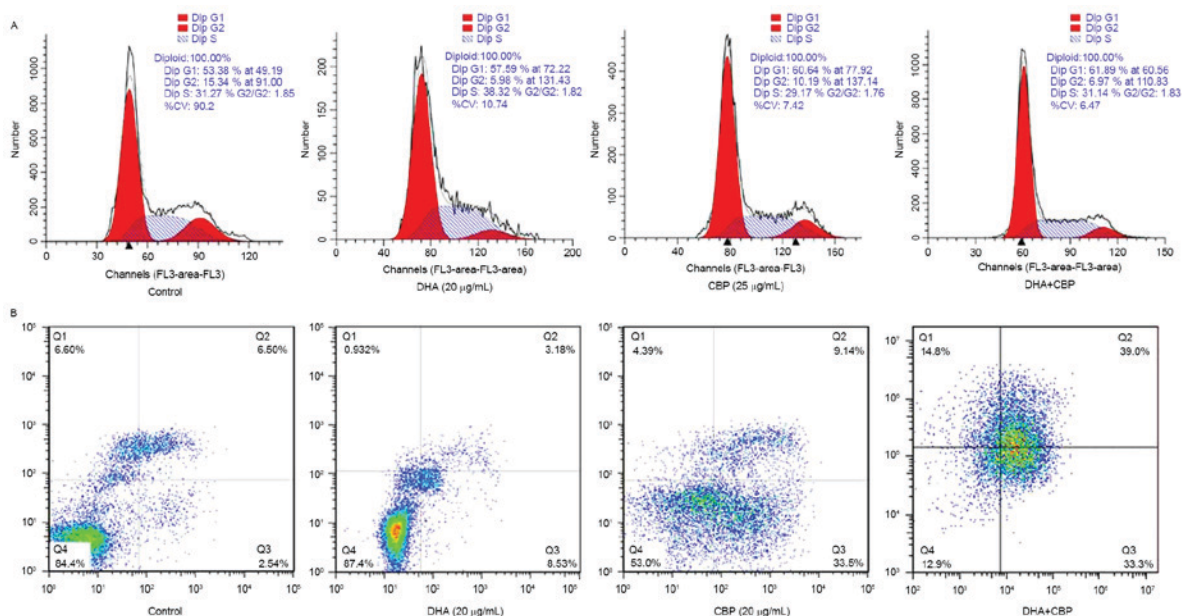


Figure 2. The effect of DHA combined with CBP on the cell cycle distribution and apoptosis of Lewis lung carcinoma cells. (A) DHA combined with CBP triggers G₀/G₁ cell cycle arrest. Cells were treated with PBS, DHA, CBP or DHA+CBP for 24 h, followed by PI staining and fluorescence-activated cell sorting (FACS) analysis for the cell cycle profile. (B) Apoptosis was determined by Annexin V-FITC/PI double-staining analysis. Annexin V-fluorescein isothiocyanate/PI flow cytometry dot plot analysis following treatment for 24 h. The percentage of cell death was determined based on the number of cells in the top right and bottom right quadrants from a total of 1.0×10^4 cells. DHA, dihydroartemisinin; CBP, carboplatin; PI, propidium iodide; FITC, fluorescein isothiocyanate.

artesunate. Among them, DHA has the highest water-solubility and antimalarial effect (17). Besides the antimalarial effect, DHA can selectively induce the proliferation inhibition and apoptosis of certain types of tumor cell (6,18). Although these previous studies have observed the antitumor effect of artemisinin and its derivatives, the underlying mechanism has not been fully elucidated. The possible mechanisms underlying DHA-induced apoptosis may be as follows: i) The reduction of the Bcl-2/Bcl-associated X, Bcl-2/BH3-interacting domain death agonist expression ratio; ii) reducing the expression level of survivin; iii) increasing the intracellular Ca²⁺ concentration of tumor cells; iv) activating the death receptor- and mitochondrion-mediated caspase-dependent signaling pathways (6,19-22). Similar to a previous report, the results of the present study revealed that the proliferation of LLC cells was inhibited by DHA by the induction of apoptosis (23). In addition to this result, our previous studies demonstrated that DHA is not only able to inhibit tumor growth, lymphangiogenesis, and the nodal and lung metastasis of LLC transplanted tumor by decreasing VEGF-C expression, but also capable of apoptosis induction and the inhibition of the migration and

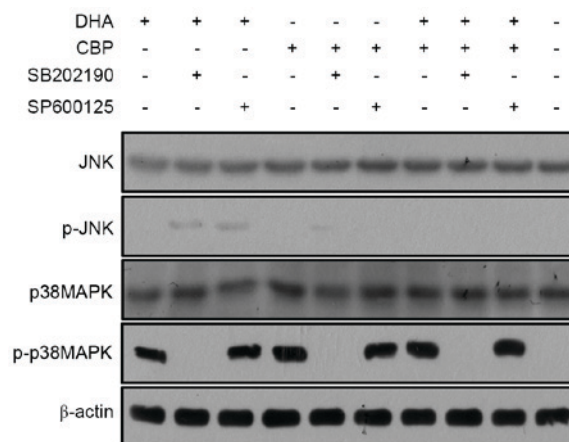


Figure 3. Potential molecular mechanisms underlying the DHA sensitization of LLC cells to CBP-induced apoptosis. The expression levels of p38MAPK-associated proteins were analyzed by western blotting in LLC cells untreated or treated with DHA (20 μg/ml), CBP (25 μg/ml), a specific inhibitor of p38MAPK (SB202190, 10 μmol/l) and JNK inhibitor (SP600125, 10 μmol/l). DHA, dihydroartemisinin; LLC, Lewis lung carcinoma; CBP, carboplatin; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; p, phosphorylated.

formation of tube-like structures in lymphatic endothelial cells *in vitro* (16,23). All these results imply that DHA may be a promising agent for controlling lung adenocarcinoma growth and metastasis *in vitro* and *in vivo*.

The results of the present study revealed that DHA increases the effectiveness of CBP by increasing the rate of apoptosis. Cisplatin, CBP and oxaliplatin are the mainstays of lung cancer chemotherapy; however, the acquired drug resistance of tumor cells and the cumulative side effects of these agents present serious clinical obstacles (24). Resistance and evasion of apoptosis are critical factors that contribute to carcinogenesis and drug resistance (25). Therefore, it is necessary to investigate novel agents and mechanisms of action to enhance the effects of therapy or sensitize cancer cells to apoptosis. The present study focused on the anticancer activities of DHA combined with CBP and revealed that DHA enhanced the inhibitory effects of CBP on LLC cells. The combined treatment of DHA and CBP induced an increase in apoptosis, compared with that produced by either compound alone. These results indicated that DHA may inhibit the growth of lung adenocarcinoma cells by increasing their apoptosis, particularly in combination with CBP. In a previous study, it was demonstrated that DHA markedly inhibited the *in vitro* and *in vivo* growth of ovarian carcinoma alone or in combination with CBP, and that it enhanced the therapeutic effect of CBP by increasing apoptosis (6). Furthermore, Feng *et al* (10) revealed that DHA potentiates the anticancer effect of cisplatin via the inhibition of mTOR in cisplatin-resistant ovarian cancer cells. Zhao *et al* (9) demonstrated the synergistic effect of a treatment combining gemcitabine with DHA in inducing the apoptosis of A549 cells via the Bcl2 antagonist/killer 1-mediated intrinsic and the Fas-caspase-8-mediated extrinsic signaling pathways. Together with the results of the present study, it has been indicated that DHA may be an effective anticancer drug, particularly in combination with conventional chemotherapeutic agents.

Furthermore, the present study demonstrated that p38MAPK activation may partially mediate the apoptosis of LLC cells induced by DHA combined with CBP. In 1994, Han *et al* (26,27) cloned p38MAPK from a murine liver cDNA library and detected the expression of p38MAPK mRNA in murine macrophages, T cells and B cells. p38MAPK is usually activated by ultraviolet radiation, hyperosmolarity, arsenate compounds, heat shock, H₂O₂, cytokines [including interleukin-1 and tumor necrosis factor- α (TNF- α)] and other physiological stresses. Following translocation from the nucleus to the cytoplasm, it initiates the activity of corresponding transcription factors (28). The role of p38MAPK in cell survival and apoptosis has been extensively examined; however, the results of these studies are inconsistent. For example, p38MAPK activation was previously demonstrated to induce apoptosis in non-tumor cells, including nerve cells and fetal brown adipocytes (29,30) and tumor cells (31,32). However, other studies demonstrated that p38MAPK did not affect apoptosis (33) or inhibited apoptosis (34-36). In our previous study, p38MAPK phosphorylation was detected in TNF- α -treated rat glioma C6 cells (37). In various types of cell line, DHA induced apoptosis by activation of signaling pathways, including the Wnt/ β -catenin, NF- κ B and caspase signaling pathways. Lu *et al* (7) revealed that DHA-induced apoptosis is dependent on iron and p38MAPK activation, but

not reactive oxygen species, JNK or ERK activation in HL-60 cells. The present study demonstrated a p-p38MAPK signal was associated with apoptosis induced by DHA combined with CBP in LLC cells. Following treatment with SB202190 and DHA simultaneously, no p38MAPK signal was detected by western blotting. The simultaneous treatment of SP600125 and DHA did not affect apoptosis. These results suggested that p38MAPK, and not JNK, serves a role in apoptosis induced by DHA and CBP in LLC cells.

In conclusion, the present study demonstrated that DHA potentially induces growth inhibition and apoptosis in lung adenocarcinoma cells and enhances the therapeutic effect of CBP *in vitro* by increasing apoptosis, which may be mediated by the activation of p38MAPK signaling. Together with previously reported findings, these results will help to improve the understanding of the mechanisms underlying DHA as a novel anticancer agent. Furthermore, the present study provides a basis for future clinical investigations of DHA in patients with lung cancer, used alone or in combination with conventional anticancer drugs. *In vivo* experiments are also required to verify the conclusion of the present study.

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