Original Paper

Dihydroartemisinin and Curcumin Synergistically Induce Apoptosis in SKOV3 Cells Via Upregulation of MiR-124 Targeting Midkine

Jiaojiao Zhao^a Yuchen Pan^a Xiujun Li^{a,c} Xuefang Zhang^a Yaxian Xue^a
Tingting Wang^a Shuli Zhao^b Yayi Hou^{a,d}

^aThe State Key Laboratory of Pharmaceutical Biotechnology, Division of Immunology, Medical School, Nanjing University, Nanjing, ^bGeneral Clinical Research Center, Nanjing First hospital, Nanjing Medical University, Nanjing, ^cDepartment of Obstetrics and Gynecology, The Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing, ^dJiangsu Key Laboratory of Molecular Medicine, Nanjing University, Nanjing, PR China

Key Words

Dihydroartemisinin • Curcumin • Midkine • miR-124 • Ovarian cancer

Abstract

Background/Aim: Women with advanced ovarian carcinoma are less likely to receive platinum-based chemotherapy and surgery due to a greater risk of cytotoxicity and poorer outcomes. We attempted to improve a promising therapy against ovarian cancer by using a combination of dihydroartemisinin (DHA) and curcumin (Cur). **Methods:** Human ovarian cancer SKOV3 cells were treated with DHA, Cur alone, or a combination of both. The viability of SKOV3 cells was measured by Cell Counting Kit-8 (CCK-8) and a colony formation assay. The cell cycle and apoptosis of SKOV3 cells were monitored by flow cytometry. The mRNA and protein expression levels of target genes were respectively examined by qRT-PCR and western blot. The biological effects of miR-124 on midkine (MK) were verified by a luciferase activity analysis. **Results:** Combined treatment of DHA and Cur synergistically decreased cell viability, arrested cell cycle, and promoted apoptosis in SKOV3 cells. Moreover, it significantly attenuated the expression of oncogene MK and synergistically upregulated the expression of miR-124. Furthermore, miR-124 was verified to bind directly to the 3'-untranslated region of MK mRNA, resulting in mRNA degradation and reduced MK protein levels. The combination of DHA with Cur significantly inhibited tumor growth in xenograft nude mice without obvious toxicity. **Conclusion:** Co-treatment with DHA and Cur exhibited a synergistic anti-tumor effect on SKOV3 cells both *in vitro* and *in vivo*.

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Yayi Hou
and Shuli Zhao

The State Key Laboratory of Pharmaceutical Biotechnology, Division of Immunology, Nanjing University, and General Clinical Research Center, Nanjing First hospital, Nanjing Medical University, Nanjing, (China); E-Mail yayihou@nju.edu.cn/shulizhao79@163.com

Introduction

Ovarian cancer is the seventh most common cancer among women and one of the most lethal female cancers worldwide. In current clinical practice, chemotherapy and surgery, and sometimes radiotherapy, are considered standard treatments for ovarian cancer, regardless of the cancer subtype [1]. The major reason for treatment failure is uncertain. It may be due to a lack of early detection, inappropriate chemotherapy, geriatric syndromes, undertreatment, or other reasons [2, 3]. There is therefore a pressing need to explore a promising strategy to optimize the treatment of ovarian cancer in older patients.

Dihydroartemisinin (DHA), a derivative and active metabolite of artemisinin, is isolated from the Chinese medicinal herb, *Artemisia annua*. It has been recommended by the World Health Organization as a first line anti-malarial therapy [4]. In addition to its potent anti-malarial activity, emerging studies have shown that DHA may also be a potential chemopreventive drug, exhibiting strong antitumor activity in various human cancer cells by blocking cell proliferation [5], arresting cell cycle [6], promoting apoptosis [7], and inhibiting angiogenesis and metastasis [8, 9]. In addition, DHA can potentiate the anticancer effect of chemotherapeutic agents and photodynamic therapy to induce cell apoptosis in various kinds of cancers [10-13]. Curcumin (Cur) is a well-known dietary polyphenol extracted from the rhizome of the plant *Curcuma longa*. Multiple studies have suggested that Cur is a promising agent possessing excellent anti-inflammation, antioxidation and anticancer activities [14-18]. Besides its capacities to directly inhibit cell viability, induce apoptosis, and inhibit migration and metastases *in vitro* and *in vivo*, Cur could synergistically enhance the radio-sensitivity and anticancer effect of some chemotherapeutic agents through regulation of diverse non-coding RNAs in different cancers, including ovarian cancer [19-21]. These data suggest that DHA and Cur may be potential alternative medicines for the clinical treatment of ovarian cancer.

Midkine (MK), a heparin-binding growth factor, is significantly overexpressed in multifarious cancers compared with healthy normal samples [22]. In general, MK expression frequently elevates with advancing tumor stage and closely correlates with poor prognosis. It was reported that blockade of MK suppressed tumorigenesis and tumor development, and an antisense oligonucleotide targeted to MK likewise repressed tumor formation in nude mice [23, 24]. We previously also found that MK was highly expressed in the gastric tumor tissues of Chinese patients and patients with malignant effusions [25, 26]. Both siRNA targeting MK gene and the conjugate (a single-chain variable fragment against human MK-Doxorubicin) inhibited gastric cancer cell growth and induced apoptosis [27, 28]. MK is therefore an attractive molecular target for cancer therapy.

Although DHA and Cur, as effective traditional Chinese medicines, both have excellent anticancer properties without any side effects, the combination effect of these compounds are rarely investigated. In this study, we showed that a combination of DHA and Cur synergistically decreased cell viability, arrested cell cycle and induced cell apoptosis in ovarian cancer SKOV3 cells. The cytotoxicity of DHA and Cur co-treatment was verified to attribute to the upregulation of miR-124 by targeting MK. Moreover, the combination treatment also made an important contribution to the antitumor effects without any toxicity *in vivo*. Taken together, these data showed that the combination of DHA and Cur may be a potent anticancer strategy for the treatment of ovarian cancer, especially in older patients.

Materials and Methods

Cell culture and drugs

The human ovarian cancer cell line SKOV3 was cultured in complete Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Gibco, Life Technologies) with antibiotics (penicillin 100 U/ml, streptomycin 0.1 mg/ml, Invitrogen, Life Technologies) and 10% fetal bovine serum (FBS, Life Technologies) at 37 °C in a 5% CO₂ incubator. Cells were grown in monolayer and passaged routinely 2~3 times a week.

Cell viability

The cell viability of SKOV3 was assessed using the Cell Counting Kit-8 (CCK-8) assay. Briefly, cells were seeded at a density of 5×10^3 cells per well in 96-well plates in quadruplicate and incubated in a 5% CO₂ atmosphere at 37 °C before treatment with drugs. After removing the culture medium, cells were treated with fresh medium containing different concentrations of DHA and/or Cur (Sigma-Aldrich, dissolved in DMSO) and incubated for 24 h or 48

h. The supernatants were then discarded; fresh medium was incorporated with 10 µl of WST-8 reagent from CCK-8 (Dojindo, Kumamoto, Japan), and the cells were incubated for another 4 h at 37 °C. Absorbance was determined with a multi-well spectrophotometer (BioTek, VT, USA) at 450 nm. Data were analyzed by CompuSyn software with combination index (CI) values based on the median-effect principle and Chou-Talalay equation to determine whether the combination was synergistic [29]. The combined effects of the two compounds can be summarized as follows: CI < 1, CI = 1, CI > 1 indicate synergistic, additive and antagonistic effects, respectively.

Isolation of total RNA and Quantitative RT-PCR

Total RNA was extracted from collected cells using TRIzol (Invitrogen, Carlsbad, CA, USA). Both miRNA and mRNA were then reverse transcribed to cDNA using the Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas) according to the instructions. The expression of mature miRNAs and potential target genes, with U6 or GAPDH serving as an endogenous control, were measured by qRT-PCR with SYBR Green PCR Kit (Bio-Rad) on the Applied Biosystems StepOne-Plus Real-Time PCR System (Applied Biosystems, Foster City, CA). The relative expression ratio of target genes was calculated by the 2^{-ΔΔCT} method; the sequences of qRT-PCR primers are provided in Table 1.

Flow cytometric analysis

For cell cycle analysis, SKOV3 cells were harvested, washed twice with cold phosphate-buffered saline (PBS), and were fixed with ice-cold 70% ethanol overnight at -20 °C. Cells were then washed twice with PBS and incubated with 0.2 ml PBS containing 50 µg/ml propidium iodide (PI), 0.1% Triton X-100 and 50 µg/ml DNase-free RNase (Sigma-Aldrich, St. Louis, MO, USA) at room temperature in the dark for 15 min. After detection by FACS Calibur FCM (BD, CA, USA), data were analyzed using Mod Fit LT 3.0 software.

Cell apoptosis analysis was conducted by Annexin V-FITC analysis. Briefly, cells were seeded for 24 h and then treated with DHA and/or Cur for another 48 h. To measure Caspase 3 activity, cells were pretreated with Caspase 3 inhibitor Ac-DEVD-CHO 50 µM for 30 min. The cells were then harvested, washed twice with PBS, stained with Annexin V-FITC and PI in binding buffer, and detected by FCM after 15 min incubation at room temperature in the dark. The early apoptotic cells (Annexin V positive only) and late apoptotic cells (Annexin V and PI positive) were quantified.

Western blotting (WB)

Cells with various treatments were lysed in an ice-cold RIPA lysis buffer (Beyotime), centrifuged to collect supernatants, and quantitated by BCA Protein Quantification Kit (Vazyme Biotech Co., Ltd.). Equal amounts of proteins were then fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membrane (Bio-Rad, Hercules, CA), blocked in 5% BSA at room temperature for 1 h, and immunostained with primary antibodies (anti-Bax, Bcl-2, Caspase 3, Cyclin A, Cyclin B and CDK1 antibodies, Cell Signaling Technologies; anti-β-actin antibody, Bioworld; anti-MK antibody, Abcam) at 4 °C overnight and secondary antibodies (ThermoFisher Scientific) at room temperature for 1 h. The expression of proteins was then visualized with ECL Plus western blotting detection reagents (Millipore, USA).

Table 1. Sequence of forward and reverse primers used for PCR amplification

Gene	Primer sequence
GAPDH	5'-GCACCGTCAAGGCTGAGAAC-3' 5'-TGGTGAAGACGCCAGTGGA-3'
U6	5'-CTCGCTTCGGCAGCACA-3' 5'-AACGCTTCACGAATTTGCGT-3'
miR-124	5'-ACACTCCAGCTGGG TAAGGCACGCGGTG-3' 5'-TGGTGTCTGGAGTTCG-3'
MK	5'-CGCGGTGCGCAAAAGAAAAG-3' 5'-TACTTGCAGTCGGCTCCAAAC-3'

ELISA assay for MK in cell culture media

MK in cell culture supernatant was measured via an enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Abcam) according to the manufacturer's instructions. Briefly, after SKOV3 cells were treated with DHA, Cur, or both for 48 h, cell culture media were collected and centrifuged at 2,000 × g for 10 min to remove debris. Aliquots of 50 µl diluted samples were then measured according to the manufacturer's instructions, and the final data were obtained from the detected OD at 450 nm.

Luciferase activities analysis

For luciferase activity analysis, human 293-T cells were cultured in 24-well plates and transfected with 0.5 µg of either wide-type or mutant psiCHECK™2 Vector containing both firefly luciferase and renilla luciferase, together with miR-NC, miR-124 mimic, inhibitor-NC and miR-124 inhibitor (RiboBio, Guangzhou, China). Transfection was performed using Lipofectamine 3000 reagent (Invitrogen Life Technologies). The cell lysates were analyzed for luciferase activity 20 h post-transfection by the Dual Luciferase Reporter Assay (Promega) according to the manufacturer's instructions.

Nude mice xenograft tumor assay

For the nude mice xenograft tumor assay, 2×10^7 SKOV3 cells were suspended in 100 µl of sterile PBS were injected subcutaneously into the right flank of female BALB/c nude mice (4-6 weeks). When tumors reached an average size of 50 mm³, mice were randomized into four groups and intraperitoneally injected with different treatments (n = 6): vehicle alone (saline), DHA alone (30 mg/kg), Cur alone (20 mg/kg), or a combination of DHA + Cur (30 mg/kg + 20 mg/kg) every two days for about 5 weeks. The body weight of the mice and the two perpendicular diameters (L and W) of tumors were recorded; tumor size was calculated according to the formula: V (volume) = (LW²) / 2, where "L" represents the largest length and "W" represents the smallest width. All animal work was approved by the Animal Care Committee of Nanjing University in accordance with Institutional Animal Care and Use Committee guidelines.

Statistical analysis

All experiments were repeated at least three times. One-way ANOVA followed by the Student's t-test were used to analyze the statistical significance among groups, and $P < 0.05$ was considered statistically significant. Graphics were built using GraphPad prism software (www.graphpad.com).

Results

DHA and Cur synergistically decrease the cell viability of SKOV3

To determine the antitumor effects of DHA and Cur, SKOV3 cells were treated with DHA and/or Cur at different concentrations, viability was measured by the CCK-8 assay, and CI value was determined. As shown in Fig. 1A and B, SKOV3 cell viability was inhibited by DHA, Cur, and their combination in a dose- and time-dependent manner. Moreover, compared to single drug application, the combination of DHA and Cur at a constant ratio of 2:1 significantly decreased the cell viability with a synergistic effect (CI < 1), except at the highest concentrations as indicated in Fig. 1C. A long-term colony formation assay in SKOV3 cells also verified that co-treatment with DHA and Cur prominently caused an irreversible cytotoxic effect compared to single DHA or Cur treatment (Fig. 1D and E). In addition, we also tested the cytotoxicity of DHA and/or Cur in other two ovarian cell lines, ES-2 and A2780. As showed in Fig. 1C, G and I, there was different effects in different cell lines; the synergistic effect was the highest in SKOV3 cells, medium in ES-2 cells, and lowest in A2780 cells. We therefore used SKOV3 cells to explore the specific mechanisms.

Combination of DHA and Cur synergistically arrests cell cycle

To explore the reason underlying the decreased cell viability, SKOV3 cells were treated with DHA and/or Cur, and the cell cycle was detected. The results showed that DHA could induce a moderate accumulation in S and G2/M phases and a reduction in G0/G1 phase (Fig. 2A and B), whereas the proportion of SKOV3 cells in the S and G2/M phases was significantly increased after the combination treatment. To confirm these phenomena, WB analysis of S

Fig. 1. DHA and Cur synergistically decrease the cell viability of SKOV3. (A) Cell viability is measured after SKOV3 are treated with indicated concentration of DHA and/or Cur for 48 h. (B) Cell viability of SKOV3 is synergistically inhibited by DHA (20 μ M) and Cur (10 μ M) in a time dependent manner. (C) The Fa-CI plot shows the CI value for each fractional effect of SKOV3 cells. The curves are generated using CompuSyn software. $CI < 1$, $CI = 1$, $CI > 1$ indicates synergistic, additive and antagonistic effects, respectively. (D) DHA and/or Cur inhibit the colony formation in SKOV3 cells. (E) Statistical analysis about the effect of DHA and/or Cur on the colony formation. (F, H) DHA and Cur decrease the cell viability of ES-2 and A2780. Cell viability of ES-2 and A2780 is measured after cells are treated with indicated concentration of DHA and/or Cur for 48 h. (G, I) The Fa-CI plot shows the CI value for each fractional effect of ES-2 and A2780 cells. All results are from three independent experiments. The values presented are the means $\pm$ SD for each group. * $P < 0.05$ and ** $P < 0.01$ vs. corresponding control.

Fig. 2. Combination of DHA and Cur synergistically arrests cell cycle. (A) The distribution of cell cycle are detected by FCM with PI staining and calculated using ModFit LT 3.0 software. (B) Quantified results of the cell cycle in SKOV3 cells. (C) DHA and Cur synergistically upregulate the expression of S and G2/M associated proteins in SKOV3 cells. All results are from three independent experiments. The values presented are the means $\pm$ SD for each group. * $P < 0.05$ and ** $P < 0.01$ vs. corresponding control.

and G2/M phase-related proteins Cyclin A, Cyclin B and CDK1 were performed. As indicated in Fig. 2C, the levels of Cyclin A/B proteins that control the transition of S and G2/M phases were nearly unchanged or increased slightly in the DHA and Cur alone treatment groups, but these two protein levels dramatically increased in the combination group. Taken together, these results suggest that the combination of DHA and Cur may synergistically induce the arrest of S and G2/M cell cycle in SKOV3 cells.

Fig. 3. Combined treatment of DHA and Cur synergistically promotes cell apoptosis independent of Caspase 3 activity (A) Representative results of the synergistic apoptosis effect of DHA and Cur on SKOV3 cells. (B) Quantitative analysis of the early and late apoptosis rate in SKOV3 cells of A. (C) WB are performed to verify the effects of DHA and/or Cur on the apoptosis-related proteins expression. (D) The Caspase 3 activity of SKOV3 cells with different treatment for 24 h. All results are from three independent experiments. The values presented are the means $\pm$ SD for each group. * $P < 0.05$ and ** $P < 0.01$ vs. corresponding control.

Combined treatment of DHA and Cur synergistically promotes cell apoptosis independent of Caspase 3 activity

To explore the anti-tumor properties of combined DHA and Cur, cell apoptosis was measured. Results showed that DHA or Cur alone treatment caused limited apoptosis in SKOV3 cells, while co-treatment with DHA and Cur resulted in extensive apoptosis (Fig. 3A and B). WB analysis of the apoptotic-related proteins showed that the expression level of the pro-apoptotic protein Bax was nearly unchanged, while the activity of Bcl-2 was markedly decreased after treatment with combination of DHA and Cur, compared to single treatment and untreated controls (Fig. 3C). Surprisingly, Caspase 3, a marker of late apoptosis, was not changed and the activity of it in the single drug or co-treatment groups was relatively lower than control (Fig. 3D). Pre-treatment with the Caspase 3 inhibitor also did not show any observable cell apoptosis in any group (Fig. 3A). These results suggest that cell apoptosis induced by DHA and/or Cur may be independent of Caspase 3 pathway.

DHA and Cur synergistically downregulates the expression and secretion of MK, a direct target of miR-124

MK is reported to be involved in various tumorigenesis and tumor development processes. To address the antitumor pharmacological mechanism of DHA and Cur against SKOV3 cells, MK levels were investigated. The results showed that the combination of

Fig. 4. DHA and Cur synergistically downregulate the expression and secretion of MK, a direct target of miR-124. (A, B) DHA and Cur synergistically decrease the mRNA and protein level of MK after 48 h. (C) Combination of DHA and Cur synergistically contribute to the decline secretion of MK into culture media after SKOV3 cells are exposed for 48 h. (D) MiR-124 is synergistically upregulated by DHA and Cur in SKOV3 cells after 24 h. (E) The fold change of miR-124 after SKOV3 cells are transfected with miR-124 mimic and miR-124 inhibitor. (F, G) The mRNA and protein level of MK are regulated after SKOV3 cells transfected with miR-124 mimics and miR-124 inhibitor for 48 h. (H) Schematic of the potential miR-124 binding sites containing 3' UTR of MK. (I) A luciferase-based activity is performed to evaluate the effect of direct binding of miR-124 to the putative target site on the 3' UTR of MK. All results are from three independent experiments. The values presented are the means $\pm$ SD for each group. * $P < 0.05$ and ** $P < 0.01$ vs. corresponding control.

DHA and Cur synergistically attenuated MK levels of mRNA and protein as well as protein secretion (Fig. 4A, B, and C).

MicroRNAs (miRNAs) are non-coding small RNAs, and their expression is often deregulated in human tumors, leading to cancer development. MK was shown to be downregulated in the DHA + Cur co-treatment group, so we further attempted to screen its putative upstream miRNA by computational prediction via three high-confidence microRNA-mRNA interaction databases: TargetScan, miRanda, and starBase. Although several miRNAs were directly upstream of MK, our results showed that among the predicted

Fig. 5. Combination of DHA with Cur-induced SKOV3 cell apoptosis is modulated by miR-124-mediated MK degradation. (A, B) MRNA and protein level of MK are analyzed in SKOV3 cells transfected with three siRNA fragments. (C) Knockdown of MK aggravates the apoptosis of DHA + Cur treated cells. (D) Quantitative analysis of the apoptosis rate of C. (E) Addition of exogenous MK reverses the apoptosis of DHA + Cur treated cells. (F) Quantitative analysis of the apoptosis rate of E. (G) Pre-treatment with miR-124 mimic is accompanied with a decrease expression of MK and miR-124 inhibitor somewhat reverses it. (H) MiR-124 overexpression suppresses the cell viability of SKOV3 cells after DHA and Cur co-treatment for 48 h. All results are from three independent experiments. The values presented are the means $\pm$ SD for each group. * $P < 0.05$ and ** $P < 0.01$ vs. corresponding control.

miRNAs, only miR-124 was synergistically upregulated by co-treatment with DHA and Cur (Fig. 4D). Interestingly, miR-124 had been reported to be down-regulated in ovarian cancer specimens, as well as in cell lines [30]. To further confirm the relationship between MK and miR-124, SKOV3 cells were transfected with miR-NC, miR-124 mimic, inhibitor NC, and miR-124 inhibitor; MK mRNA and protein levels were analyzed. The results showed that miR-124 could downregulate both mRNA and protein levels of MK, while the miR-124 inhibitor reversed the MK expression, indicating that MK was indeed regulated by miR-124 (Fig. 4E, F, and G). To verify that miR-124 directly targeted MK via a molecular mechanism, a luciferase assay was adopted in 293-T cell models transfected with the psiCHECK™2 Vector with a wild-type or mutant full length 3'-UTR of MK. As Fig. 4H and I indicate, overexpression of miR-124 mimic significantly suppressed the luciferase activity of reporter genes containing 3'UTR of MK compared with the control, while the miR-124 inhibitor hardly affected the luciferase activity, verifying that MK was a direct target of miR-124.

Fig. 6. Combination of DHA with Cur synergistically inhibits tumor formation *in vivo*. (A) DHA and/or Cur inhibits cell proliferation in solid tumor by immunohistochemistry with Ki67. (B) Quantitative analysis of the cell proliferation rate of A. (C, D) Tumor volumes in DHA and/or Cur treatment groups. (E) Tumor weights in DHA and/or Cur treatment groups. (F) Body weight of the tumor-bearing mice in DHA and/or Cur treatment groups. The values presented are the means $\pm$ SD for each group. * $P < 0.05$ and ** $P < 0.01$ vs. corresponding control.

Combination of DHA with Cur-induced SKOV3 cell apoptosis is modulated by miR-124-mediated MK degradation

We investigated whether the DHA + Cur-induced SKOV3 cell apoptosis are related to miR-124-mediated MK dysfunction. First, we explored cell apoptosis in MK deletion and addition conditions. As shown in Fig. 5A and B, si-MK3 had the most visible MK silencing effect; thus, we choose it for the next experiments. The results showed that knockdown of MK further aggravated apoptosis (Fig. 5C and D), whereas the addition of exogenous cytokine of MK reversed it (Fig. 5E and F). In addition, SKOV3 cells were pre-treated with miR-124 mimic and miR-124 inhibitor to mimic a miR-124 gain and loss of function, and then were further co-treated with DHA and Cur to explore the relationship between miR-124 and MK. The results showed that the group pre-treated with miR-124 mimic further decreased the expression of MK (Fig. 5G), whereas miR-124 inhibitor partially overturned this phenomenon compared with control. Furthermore, overexpression of miR-124 mimic before co-treatment of DHA + Cur reduced cell vitality, and miR-124 inhibitor slightly reversed it (Fig. 5H). The data suggest that DHA + Cur induced SKOV3 cell apoptosis was at least partially via miR-124 mediated MK degradation.

Combination of DHA with Cur synergistically inhibits tumor formation in vivo

To investigate whether the combination of DHA and Cur has the same elimination effect on tumors *in vivo*, a heterologous SKOV3 tumor model was established. DHA alone (30 mg/kg), Cur alone (20 mg/kg), and DHA + Cur (30 mg/kg + 20 mg/kg) were administered by intraperitoneal injection. The cell proliferation in solid tumor was measured by Ki67. As shown in Fig. 6A and B, the suppression of cancer cell proliferation was exhibited more prominently in the combination group than in either the DHA or Cur alone group. In addition, the combined treatment of DHA and Cur significantly inhibited the tumor growth compared to control or single drug treatment (Fig. 6C, D, and E). Moreover, the mean body weight of all drug-treated groups was hardly changed in comparison with the control group, which suggests that the combination of DHA + Cur may not cause serious adverse effects *in vivo* (Fig. 6F).

Discussion

For the treatment of ovarian cancer, a standard recommendation is a primary chemotherapy followed by surgical resection [31]. This therapy, however, may be difficult for some patients with advanced ovarian carcinoma, with a greater incidence of poor prognosis. Therefore, novel therapeutic agents that specifically, efficiently, and safely treat this condition are needed. DHA and Cur are among the most popular nutritional supplements. DHA is available inexpensively and Cur supplements are currently available worldwide [32]. We therefore tentatively suggest that a combination of DHA and Cur, two active ingredients in Chinese herbs, might be an alternative for treatment of patient with ovarian cancer. Our results presented in this paper demonstrate that DHA and Cur synergistically aggravated the apoptosis of SKOV3 cells and might be involved in upregulation miR-124 targeting MK.

In this study, DHA and Cur alone inhibited the cell viability of SKOV3 cells in a dose- and time-dependent manner; the combination treatment of DHA and Cur was more effective than either single agent, demonstrating a synergistic effect. As our data demonstrates, while DHA alone could more effectively arrest cell cycle at S and G2/M, Cur further potently induced cell apoptosis, and the combination of both contributed to a considerable decrease in cell viability. Previous studies revealed that DHA and Cur treatment alone delayed the growth of ovarian cancer, including a dose-dependent G2/M arrest [33, 34]. In addition, targeting the Bcl-2 family-mediated apoptosis components was an important mechanism for the antitumor activity of DHA in cancer cells, and Cur could potentiate the anticancer effect of chemotherapy for cell apoptosis [35, 36]. Consistent with previous reports, our results also indicated that elevated apoptosis in the co-treatment of DHA and Cur group

was accompanied by a decrease of Bcl-2. Nevertheless, the apoptosis induced by DHA, Cur or the DHA + Cur combination failed to activate Caspase 3. Pre-treatment with Caspase 3 inhibitor did not inhibit cell apoptosis in any group, indicating that the apoptotic pathway induced by DHA and Cur was not via activation of Caspase 3, and other apoptotic pathways may contribute to the cell apoptosis.

As a biomarker in several clinical oncology settings, MK has already been proven to have utility for initial cancer screening and recurrence monitoring. Numerous studies have shown that miRNAs are aberrantly expressed with tumorigenesis, and many miRNAs are downregulated in ovarian cancer [37], among which, miR-124 has been widely reported to function as a tumor suppressor by regulating cell proliferation, invasion and apoptosis. In our study, depletion of MK by siRNA further accelerated the apoptosis of SKOV3 cells, and exogenous addition overturned the phenomenon in the co-treatment group. Overexpression of miR-124, which was synergistically upregulated by DHA and Cur, also attenuated the expression of MK and caused decreased cell viability in SKOV3 cells. The miR-124 inhibitor, however, only slightly reversed the MK expression in mRNA level, and the protein level was nearly unchanged, which meant that other factors might be also involved in the regulation of MK in SKOV3 cells. In addition, our study also found that, when co-treatment with DHA and Cur, the MDR1 coding P-gp responsible for transporting a wide range of anticancer agents was also attenuated. Silencing of MK by siRNA and overexpression of miR-124 both caused a decrease of MDR1 (data not shown). Therefore, we tentatively put forward that the combination treatment of DHA and Cur may also have excellent anti-tumor properties in SKOV3 cells by destroying the P-gp function of excreting drugs, causing additional cytotoxicity. More importantly, compared to control and single drug treatment, the xenograft nude mice tumor model also demonstrated that co-treatment of DHA + Cur caused significant tumor retardation without any side-effects. These results indicated that DHA and Cur combination treatment was a safe therapeutic regimen.

In conclusion, our studies revealed that combination treatment with DHA and Cur synergistically exhibited prominent anti-tumor activity by inhibiting cell viability, arresting cell cycle and promoting cell apoptosis both *in vitro* and *in vivo*. The underlying molecular mechanisms might be through upregulation of miR-124 targeting MK for efficient cytotoxicity to SKOV3 cells. As both DHA and Cur are commercially available, therapeutically effect and most importantly, remarkably safe, it can be considered as a promising therapeutic strategy for ovarian cancer in the elderly.

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Disclosure Statement

The authors declare no conflict of interest.

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