Dihydroartemisinin triggers c-Myc proteolysis and inhibits protein kinase B/glycogen synthase kinase 3β pathway in T-cell lymphoma cells

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Abstract. Recent studies have revealed a positive therapeutic effect of dihydroartemisinin (DHA) on tumor cells. However, the underlying mechanism of this has not yet been elucidated. The present study examined the potential therapeutic role and mechanism of DHA in T-cell lymphoma cells. It was revealed that DHA inhibited the proliferation of Jurkat and HuT-78 T-cell lymphoma cells in a concentration- and time-dependent manner. Furthermore, DHA reduced c-Myc protein expression at the transcriptional level, and induced the phosphorylation of c-Myc and the degradation of its oncoprotein levels. DHA treatment resulted in decreased phosphorylation of protein kinase B (Akt) and glycogen synthase 3β (GSK3β) in T-cell lymphoma cells. In addition, DHA treatment induced cell apoptosis, which was accompanied by an increased ratio of Bax/Bcl-2. Taken together, the results of the present study suggested that DHA may exert its antitumor role by accelerating c-Myc proteolysis and inhibiting the Akt/GSK3β pathway in T-cell lymphoma cells.

Introduction

T-cell lymphoma accounts for 9.7% of all lymphoid malignancies, with an incidence rate of 1.17 cases per 100,000 people in Hong Kong (1). T-cell lymphoma has distinct clinical features and a complicated epidemiology (2). Despite treatment with intensive chemotherapy or combined chemotherapy, patients with T-cell lymphoma continue to exhibit suboptimal outcomes and a poor disease progression (3,4). The development of effective therapeutic treatments to improve the prognosis of T-cell lymphoma is required.

The c-Myc transcription factor is a short-lived nuclear phosphoprotein that serves a vital role in regulating cell proliferation, differentiation and apoptosis (5,6). Notably, overexpression of c-Myc has been associated with the occurrence of hematopoietic malignancies, including lymphoma and leukemia (7). Glycogen synthase kinase 3β (GSK3β) regulates c-Myc levels and activity by phosphorylating c-Myc at threonine 58 to promote its degradation by the ubiquitin-proteasome pathway in lymphoma (8). A previous study in acute lymphocytic leukemia cells revealed that GSK3β exhibits a low affinity to the stabilized c-Myc oncoprotein (7). Taken together, these results suggested that downregulation of c-Myc expression may be an effective strategy for the treatment of lymphoma.

The phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt)/GSK3β signaling pathway serves important regulatory roles in differentiation, cell survival and apoptosis (9). Akt is a critical downstream factor of PI3K and is activated through PI3K phosphorylation of Akt at serine 473 (10). Phosphorylated Akt (p-Akt) subsequently activates GSK3β, its principal physiological substrate, by phosphorylation of GSK3β at serine 9 (10). Activated Akt supported a survival signal by phosphorylating apoptotic factors, including GSK3β, and preventing cells from apoptosis (9,11,12). Previous studies have revealed that the Akt/GSK3β signaling pathway is activated to promote cell proliferation and decrease apoptosis in various types of carcinoma (13). The function of the p-Akt/GSK3β pathway is to promote proliferation and to decrease apoptosis. p-Akt is involved in tumor proliferation and a poor prognosis in several types of human cancer, and it may be an important factor in the development of lymphomas (14,15).

Dihydroartemisinin (DHA) is a compound isolated from Artemisia annua L., a Chinese medicinal herb. A few reports, including our previous study, indicated DHA as a promising drug for tumor therapy due to its antitumor activity in cancer cells (16-18). DHA significantly induced c-Myc oncoprotein degradation in tumor cells overexpressing c-Myc, a process that involved GSK3β phosphorylation of c-Myc at...
threonine 58 (19). DHA also suppressed cell proliferation via the Akt/GSK3β/cyclin D1 signaling pathway in A549 lung cancer cells and promoted the degradation of c-Myc via increased phosphorylation of c-Myc (19,20).

Based on these studies, we hypothesized that DHA exhibits antiproliferation activity in T-cell lymphoma cells and that DHA may mediate c-Myc oncprotein degradation and downregulate the Akt/GSK3β signaling pathway in T-cell lymphoma cells. Therefore, the present study examined the potential anticancer effect of DHA on T-cell lymphoma cells (Jurkat and HuT-78 cells). We also investigated the underlying mechanism of DHA was also investigated in T-cell lymphoma cells and the potential involvement of the AKT/GSK3β signaling pathway. This study may provide evidence for DHA as a possible treatment for T-cell lymphoma cells.

Materials and methods

Materials and cell culture. DHA was obtained from Chunyou Biological Technology Corporation (Shanghai, China) and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) as a stock solution of 8,000 μM at -20°C. RPMI-1640 medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) was used to dilute the stocked DHA for each experiment.

T-cell lymphoma Jurkat and HuT-78 cell lines were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Jurkat cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and HuT-78 cells were cultured in Iscove's modified Dulbecco's medium (HyClone; GE Healthcare Life Sciences) containing 20% fetal bovine serum. Medium was supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin and 2.5 mg/ml amphotericin B, and cells were incubated at 37°C in a humidified 5% CO₂ atmosphere. The control group comprised cells not exposed to DHA, while the DHA group comprised cells treated with DHA.

Cell viability assay. Cell viability assay was evaluated using Cell Counting kit-8 (CCK-8; Anhui Yiyuan Biotechnology Co., Ltd., Anhui, China). Jurkat cells were seeded at a density of 8x10⁴ cells/well in 100 μl RPMI-1640 medium supplemented with 10% fetal bovine serum and HuT-78 cells were seeded at a density of 8x10⁴ cells/well in 100 μl Iscove's modified Dulbecco's medium containing 20% fetal bovine serum in a 96-well plate (Costar; Corning Incorporated, Corning, NY, USA), and the cells were incubated for 24, 36, 48, 60 or 72 h with DHA (0, 5, 10, 20, 40 or 80 μM) added to the culture medium. At each time point, 10 μl CCK-8 was added to each well and the cells were subsequently incubated at 37°C for 2 h. Absorbance was detected at 450 nm on a microplate absorbance reader (Sunrise; Tecan Group Ltd., Männedorf, Switzerland).

Cell apoptosis assay. Jurkat (1x10⁶) were seeded onto a 6-well plate with 2 ml RPMI-1640 medium supplemented with 10% fetal bovine serum and HuT-78 (1.5x10⁶) cells were seeded onto a 6-well plate with 2 ml Iscove's modified Dulbecco's medium containing 20% fetal bovine serum followed by incubation at 37°C for 48 h. Subsequently, DHA (0 or 15 μM for Jurkat cells and 0 or 30 μM for HuT-78 cells) was added. All cells were resuspended with cold PBS (HyClone; GE Healthcare Life Sciences) and centrifuged at 447.2 x g for 5 min at room temperature. Cells were resuspended in 300 μl cold binding buffer (BD Biosciences, Franklin Lakes, NJ, USA) following the addition of 5 μl Annexin V-fluorescein isothiocyanate (BD Biosciences) for 15 min in the dark at room temperature. Propidium iodide (PI; 5 μl; BD Biosciences) was added and incubated for another 5 min, and another 200 μl binding buffer was added, followed by a flow cytometer (BD Biosciences) FlowJo software version 7.6.1 (FlowJo LLC, Ashland, OR, USA) being used to analyze each sample.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the cultured cells using RNAiso Plus (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's protocol. Pectrophotometry (Dinamica Scientific Ltd., Newport Pagnell, UK) was used to determine the quality and quantity of RNA. Total RNA was reverse transcribed into single-stranded cDNA using a Prime Script™ RT reagent kit (Takara Bio, Inc.), according to the manufacturer's protocols. Next, the reverse transcribed single-stranded cDNA was amplified by SYBR1 Premix Ex Taq II (Takara Bio, Inc.) with a CFX96 spectrofluorometric thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primers were synthesized by Tsingke Biological Technology Co., Ltd. (Beijing, China; Table I). Thermocycling conditions included a pre-incubation stage at 95°C for 30 sec, and 40 cycles of amplification stage at 95°C for 5 sec and 60°C for 30 sec, and a melt curve stage at 95°C for 15 sec, 65°C for 60 sec and 95°C for 15 sec. Relative expression of GAPDH mRNA was used as an endogenous internal control. Gene expression changes was assessed with the 2-ΔΔCq method (21).

Western blot analysis. Cells were washed with PBS and lysed with radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China), phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology) and phosphatase inhibitor cocktail I (MedChemExpress, Monmouth Junction, NJ, USA). Protein concentrations of each lysate were detected using the bicinchoninic acid method (Beyjing ComWin Biotech Co., Ltd., Beijing, China), prior to each lysate being boiled at 95°C for 5 min. Equal quantities (20 μg) of protein were separated by 10 or 12% SDS-polyacrylamide gel and electrotransferred onto polyvinylidene difluoride membranes (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The membranes were blocked for 2 h with 5% skimmed milk in TBST at room temperature (Beijing Solarbio Science & Technology Co., Ltd.), while the membranes transferred with phosphorylated protein were blocked with 5% bovine serum albumin (Beijing Solarbio Science & Technology Co., Ltd.) in TBST at room temperature for 2 h. Each membrane was incubated with primary antibodies (Abcam, Cambridge, UK; Table II) overnight at 4°C, washed three times with TBST for 15 min each time, and incubated with secondary antibody (cat. no. E-AB-1003; Elabscience Biotechnology Co., Ltd., Wuhan, China) for 1 h at room temperature. The secondary antibody was horseradish peroxidase-conjugated goat-anti-rabbit IgG.
and the dilution was 1:2,000. Subsequently, the membranes were washed three times with TBST for 15 min each time, and the immunoblots were measured with a Fusion FX7 system (Vilber Lourmat, Marne-la-Vallée, France) using a chemiluminescent horseradish peroxidase substrate (EMD Millipore, Billerica, MA, USA). Relative expression of GAPDH was calculated as an endogenous internal control for protein loading.

**Statistical analysis.** All experiments were performed at least three times. Data were analyzed using GraphPad Prism 5.01 (GraphPad Software, Inc., La Jolla, CA, USA) and are expressed as the mean ± standard deviation of at least three separate experiments. One-way analysis of variance (ANOVA) was used to compare the results of cell viability and cell proliferation, as well as to compare different time points and concentrations in Jurkat and Hut-78 cells. LSD test was used following ANOVA. All other results were evaluated using an independent-samples t-test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

*DHA decreases the viability of Jurkat and HuT-78 cells.* The present study first evaluated the effects of DHA on the proliferation of the T-cell lymphoma Jurkat and HuT-78 cell
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Jurkat and HuT-78 cells were treated with various concentrations of DHA for different time periods. Cell morphology analysis revealed that treatment with 15 µM DHA in Jurkat cells and 30 µM in HuT-78 cells damaged the integrity of the cell membrane structure and induced a marked reduction in cell density (Fig. 1A). The CCK-8 results demonstrated that DHA decreased the viability of Jurkat and HuT-78 cells in a concentration- and time-dependent manner (Fig. 1B and C). Cell viability had statistical significances in different time points/concentrations in the Jurkat cell group (all P<0.001). Additionally, cell viability had statistical significances (P<0.05) except at 36 and 48 h (P=0.53) in HuT-78 cell group. The IC50 values of DHA at 48 h for Jurkat and HuT-78 cells were 16.63 and 33.35 µM, respectively.

**DHA promotes the apoptosis of Jurkat and HuT-78 cells.** Jurkat cells were treated with 15 µM DHA, and HuT-78 cells were treated with 30 µM DHA for 48 h, and apoptosis was examined by Annexin V/PI staining. Flow cytometric analyses demonstrated that 15 µM DHA significantly induced Jurkat cell apoptosis (P<0.001). Compared with the control group, which exhibited an apoptosis rate of 1.53%, the apoptosis rate of the DHA treatment group was increased to 32.44%. The apoptosis rate of the HuT-78 cells was also significantly increased to 22.81% following administration of 30 µM DHA compared with 1.65% in the control cells (P<0.001; Fig. 2A and B).

The mRNA and protein expression levels of Bax and Bcl-2 were also evaluated by RT-qPCR and western blot analysis.
In the two cell lines, Bax mRNA and protein expression was increased while Bcl-2 mRNA and protein expression was significantly decreased following exposure to DHA compared with the control group (Fig. 2C-E). In Jurkat cells, DHA induced a 4.72-fold increase in the ratio of Bax/Bcl-2 mRNA compared with the control group and increased the ratio of Bax/Bcl-2 protein expression by over 2-fold. In HuT-78 cells treated with DHA, the ratio of Bax/Bcl-2 mRNA and protein expression increased by 6.08-fold and 3.39-fold, respectively, compared with the control group (Fig. 2F).

**DHA enhances c-Myc phosphorylation at threonine 58 in Jurkat and HuT-78 cells.** To determine whether DHA exerted any effect on c-Myc mRNA levels, the relative expression of c-Myc mRNA was detected by RT-qPCR (Fig. 3A). It was revealed that c-Myc mRNA expression was downregulated in Jurkat cells treated with 15 µM DHA and HuT-78 cells treated with 30 µM DHA. The differences were statistically significant compared with the control groups (P<0.001).

To determine whether DHA exhibited any effect on c-Myc protein levels, c-Myc protein and c-Myc phosphorylated at threonine 58 in Jurkat and HuT-78 cells.
threonine 58 expression levels were determined by western blot analysis. The expression level of phosphorylated c-Myc at threonine 58 was increased following DHA treatment for 2 h, while the total relative expression of c-Myc oncoprotein was markedly decreased in Jurkat and HuT-78 cells following treatment with DHA for 48 h (Fig. 3B and C). The differences in expression levels were significant compared with the control groups (P<0.05). DHA did not have a significant effect on the expression level of GSK3β protein (P>0.05; Fig. 3B).

**Discussion**

T-cell lymphoma is a common hematological malignancy, and its occurrence is associated with alterations in critical genes and activation of pivotal signaling pathways (5). High expression of c-Myc is associated with the occurrence and development of lymphoma (7). In addition, the Akt/GSK3β signaling pathway serves a critical role in the regulation of tumor cellular processes, including proliferation, differentiation and apoptosis (7,9). DHA is an effective drug in treating patients with malaria (22). Recent studies have revealed that DHA has antitumor activity with few side effects in various types of tumors in vivo and in vitro (16-18,23-25). Based on the results of these aforementioned studies, the present study investigated the therapeutic role of DHA and its underlying mechanism in T-cell lymphoma cells.

In the present study, DHA inhibited the proliferation of T-cell lymphoma cells in a concentration- and time-dependent manner. The results of the present study were consistent with those of previous studies in Jurkat and ovarian cancer cells (16,18). When different concentrations of DHA were compared, it was revealed that Jurkat cells were more sensitive to DHA than HuT-78 cells. These results indicated that DHA may be a promising drug for treatment in T-lymphoma
cells. However, the effect of DHA on the patient samples and xenograft mouse model in T-cell lymphoma were not assessed in the present study; therefore, we will further investigate the effect of DHA in future in vivo studies.

The results of the present study demonstrated that DHA reduced c-Myc protein expression by two potential mechanisms. To begin with, DHA treatment caused a decrease in the c-Myc mRNA level, resulting in a decreased c-Myc protein expression. Additionally, DHA enhanced the phosphorylation of c-Myc at threonine 58, which may trigger c-Myc oncoprotein degradation in T-cell lymphoma cells by the ubiquitin proteasome system. A previous study revealed that DHA promoted c-Myc oncoprotein degradation in HL-60 and HCT116 cells (19). This suggested that DHA-induced phosphorylation of c-Myc at threonine 58 targets c-Myc for degradation. Taken together, the results of the present study suggested that DHA suppressed c-Myc protein expression at the transcriptional and post-transcriptional level, leading to inhibition of cell proliferation and induction of cell apoptosis.

The Akt/GSK3β signaling pathway serves an important role in the regulation of cell proliferation and apoptosis in cancer. The Akt/GSK3β signaling pathway is activated upon phosphorylation of Akt and GSK3β at serine 473 and serine 9, respectively, and promotes proliferation and inhibits apoptosis. Previous studies have demonstrated that DHA could inhibit the Akt/GSK3β signaling pathway in lung cancer and glioma cells (23,25). To further elucidate the mechanism of DHA treatment in T-cell lymphoma cells, the present study investigated the effect of DHA on the Akt/GSK3β signaling pathway. The results of the present study revealed that DHA could suppress the Akt/GSK3β signaling pathway through a process that involved reduction of p-Akt and p-GSK3β expression levels, but did not impact GSK3β steady-state levels.

The Bcl-2 protein is an anti-apoptotic factor that serves a critical role in apoptosis, whereas Bax functions as a pro-apoptotic effector (26). The results of the present study suggested that DHA could abrogate c-Myc-induced apoptosis (27), and that c-Myc may synergize with Akt in cell growth and proliferation (28). These studies indicated that c-Myc cooperates with Akt and Bcl-2 to promote tumor progression. These results combined with those of the present study further suggested that the DHA-induced...
apoptotic effect may also involve DHA reducing Bcl-2 expression and relieving its inhibition of c-Myc. DHA also reduces the expression of c-Myc and p-Akt, leading to the suppression of Jurkat and HuT-78 cell growth and proliferation.

In conclusion, the results of the present study suggested that DHA decreased c-Myc protein expression levels at the transcriptional level and triggered c-Myc degradation by enhancing the phosphorylation of c-Myc at Thr80-Myc by c-Myc degradation. Furthermore, DHA blocked cell proliferation by suppressing the Akt/GSK3β signaling pathway and reducing c-Myc protein expression, and DHA also induced apoptosis by increasing the ratio of Bax/Bcl-2 in T-cell lymphoma cells. The results of the present study aid in clarifying the mechanism of DHA antitumor activity in T-cell lymphoma cells.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SW, WW, XZ, CZ and HZ conceived and designed the experiments. WW, XZ, LS and YC performed the experiments and acquired the data. LS analyzed and interpreted the data regarding the cell apoptosis assay. SW, WW, XZ, CZ and HZ revised the manuscript critically for important intellectual content. SW, CZ and HZ revised the manuscript critically for important intellectual content.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declared that they have no conflict of interest.

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