Dihydroartemisinin Exhibits Antitumor Activity Toward Human Gastric Cancer Cells Through the Endoplasmic Reticulum Stress Pathway

Si Chen
Shenzhen University
Hanlin Wang
Shenzhen University
Tie Chen
Shenzhen University
Furong Deng
Shenzhen University
Hongbin Li
Shenzhen University
Lixin Xia (xialixin@szu.edu.cn)
Shenzhen University
Jian Zhang
Shenzhen University

Research

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Abstract

Background: Gastric cancer is the most fatal digestive tract tumor. The current treatment of gastric cancer often causes adverse effects. Dihydroartemisinin (DHA), a first-line antimalarial drug, is a derivative of a compound from a well-known Chinese medicinal plant *Artemisia annua*. DHA demonstrates antitumor activities toward many different types of cancer while exerts no apparent adverse effects on normal cells, making it a promising lead compound for cancer treatment. DHA induces apoptosis in Gastric cancer cell line 7901 (SGC-7901). However, the exact mechanism of this antitumor activity remains not fully explored.

Methods: A CCK-8 assay to detect cell viability with DHA treatment in gastric cancer SGC-7901. The colony formation was visualized by crystal violet staining. The DHA-treatment cells were stained by Annexin-V FITC/PI dye and then subject to cell flow cytometry. The apoptosis was further observed in the Hoechst staining assay. Real-time qPCR was conducted to detect apoptosis-related markers. Western blotting was conducted to detect the protein levels of the endoplasmic reticulum (ER) stress pathway-related proteins. KIRA6, an ER stress pathway inhibitor was applied to find out whether it could reverse the cell death.

Results: DHA induced dose-dependent apoptosis in Gastric SGC-7901 cell with an IC_{50} of about 4 mg/mL. It significantly increased the proportion of apoptotic cells in a dose-dependent pattern in the Annexin V/PI flow cytometry. Significantly higher percentages of cells with a more prominently stained nucleus were observed in the Hoechst staining assay. In qPCR assay, the mRNA level of Bcl-2 was significantly decreased while that of Bax was significantly increased in a dose-dependent manner after DHA treatment. In the western blot assay, increased Bax and Bim and decreased Caspase 9, Bcl-2 were observed. Consistently, the levels of pro-apoptotic proteins were increased while those of anti-apoptotic ones were decreased as shown in the Human Apoptosis Array assay after DHA treatment. DHA stimulated the expression of GRP78, ATF4, IRE1, CHOP, and phosphorylated c-Jun (p-c-Jun) as revealed in western blotting. The cell death caused by DHA treatment was reversed by KIRA6.

Conclusions: DHA exerts its antitumor activity on SGC-7901 cells through the IRE1/c-Jun ER stress pathway.

Introduction

Gastric cancer is one of the most common malignant tumors of the digestive tract in the world, accounting for over 1,000,000 new cases in 2018 and an estimated 783,000 deaths in 2018 [1]. Since the early stage of gastric cancer is asymptomatic, many patients, especially those from the developing countries where early screening lacks, are already at an advanced stage when the diagnosis is made, resulting in a poor 5-year survival rate of less than 30% in most countries [2]. Although great progress in radiotherapy and chemotherapy of gastric cancer has been made in the past decades, treatment of

cancer remains unsatisfactory due to many severe side effects. Therefore, it is essential to develop new effective therapies by using new lead compounds with low toxicity.

Dihydroartemisinin (DHA), a semi-synthetic derivative of Artemisinin from Chinese medicine, is an efficacious antimalarial drug [3]. Recently many studies have found that DHA exhibits antitumor effects toward many types of tumors, including gastric cancer, with low toxicity toward normal cells, making it a promising antitumor agent [4]. DHA exerts its antitumor activities toward gastric cancer through different biological processes. It prevents *Helicobacter pylori*-induced gastric carcinogenesis via inhibition of NF-κB signaling [5]. Another group's work finds out that DHA induces apoptosis in human gastric cancer cell line BGC-823 through activation of JNK1/2 and p38 MAPK signaling pathways [6]. DHA has been also found to inhibit the growth of gastric cancer cells by regulating cell cycle-related signaling [7]. By applying gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses, Liang et al. prove that DHA inhibits tumorigenesis and invasion of gastric cancer cells by regulating STAT1/KDR/MMP9 and p53/BCL2L1/CASP3/7 pathways [8]. Some groups report the involvement of ER stress pathway in DHA-induced cell death [9,10]. ER stress occurs when the protein folding capacity of the Endoplasmic Reticulum is disturbed by hostile micromilieu such as high metabolic demand and oxidative stress in tumor cells [11]. Unfolded protein response, which recovers ER homeostasis and promotes cell survival, is the major pathway of ER stress [12,13]. Its downstream sensors include GRP78, PERK, ATF6, IRE1, ATF4, CHOP and c-Jun. The hostile micro-environments, especially the hypoxic conditions produced by the highly proliferative cancer often result in ER stress. To deal with ER stress, cancer cells, unlike normal cells, have evolved ways including the unfolded protein response to help them to resolve disorders such as dysregulation of protein synthesis, folding, or posttranslational modifications. Many antitumor agents, therefore exploit the difference of ER stress levels between cancer and normal cells to exert their toxicity [14]. However, whether ER stress is involved in the cell death of DHA-treated gastric cancer cells remains unresolved.

In the current study, we found that DHA treatment induced ER stress in SGC-7901 cells. Neutralization of ER stress could suppress DHA-induced cell death. Our findings contribute to the understanding of DHA in the future therapy of gastric cancer.

Materials And Methods

Cell lines and reagents

All cell lines were purchased from the American Type Culture Collection (Manassas, Virginia, USA). SGC-7901 cells were maintained in DMEM (Hyclone, Thermo Fisher Scientific, Florence, KY, USA) with 10% FBS and 100 U/ml penicillin-streptomycin. Dihydroartemisinin (DHA) was obtained from Chengdu MUST Bio-Technology., LTD.

Cell viability assays

A Cell Counting Kit 8 (CCK-8, Dalian Meliun Biotech Co., Ltd., Dalian, China) assay was performed to measure cell viability and proliferation. Briefly, cells were seeded in 96-well plates at a concentration of 5000 cells/well and incubated in a final volume of 200 ml culture medium. Cells were treated with different concentrations of Dihydroartemisinin (0-160 mg/ml) or other compounds for 48 h to measure the IC_{50}. Cell viability was counted via the addition of CCK-8 reagent.

Colony formation assays

Cells were seeded into a 6-well plate in DMEM medium at a density of 1000 cells and cultured in a final volume of 2 ml culture medium containing DHA (0, 2, or 4 mg/ml) for 9-14 days until clones were visible to the naked eye. Colonies that contained > 30 stained cells were classified as clones. After washing, cells were fixed with 100% methanol at 4℃ for 10 min and stained with 0.05% crystal violet for 15 minutes at room temperature, and then washed twice with ddH_2O and air-dried.

Cell apoptosis assay

Cells were seeded into a 6-well plate with 7×10^5 cells/well in DMEM medium for 24 h, followed by treatment with DHA (0, 2.5, 5, 10 mg/ml) for 48h. After treatment, the numbers of cells were adjusted approximately equal in each group. The cells were washed with PBS and suspended in a solution containing 5 mL Annexin-V FITC and 5 mL PI dye (BD, San Jose, CA, USA). Following incubation in the dark for 15 min, cell apoptosis was analyzed by using Accuri C6 flow cytometry (BD, San Jose, CA, USA).

Hoechst 33258 staining assay

An apoptotic Hoechst Staining Kit (Beyotime, Shanghai, China) was employed to detect cell apoptosis. Cells were seeded in 12-well plates with a coverslip at a density of 1.0×10^5 cells per well and incubated for 48 hours. Briefly, after fixation, the cells were rinsed with PBS and stained with Hoechst 33258. Cell apoptosis was detected at 350 nm by a confocal microscope, and the tumor cell apoptotic rate was calculated.

Total RNA isolation

Total RNA was extracted from the cultured cells (7×10^5 cells) and lysed in 1 mL Trizol reagent. 200 mL chloroform was added to separate the RNA at the aqueous phase, and 500 mL isopropanol was then applied. Finally, the RNA was centrifuged at 14,000 rpm (4℃) for 15 min and the RNA was dissolved in DEPC-MQ H_2O. The concentration was measured with a Nanodrop 144 spectrometer N1000 (Thermo Fisher Scientific, Inc.).

Real-time qPCR

The extracted RNA was reverse-transcribed to cDNA using the Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). The cDNA obtained via reverse transcription was diluted 10-fold and prepared according to the SYBR Green Reagent system for real-time fluorescence quantitative assay

(Vazyme Biotech co. Ltd., Nanjing, China). Each qPCR reaction was repeated three times, and the expression of the target genes was normalized to the internal reference GAPDH. The thermal cycling of qPCR was as follows: 95°C for 2 min; 40 cycles of 95°C for 15 s and 60°C for 30 s. The mRNA expression of the target genes was analyzed using the $2^{-\Delta\Delta CT}$ calculation method. The primers are listed in the Supplementary Information.

Protein extraction and Western blot

SGC-7901 cells were collected with RIAP lysis buffer containing protease inhibitors. The cell lysates were centrifuged and the supernatants were collected. The protein concentration was determined using a BCA Protein Assay kit (Beyotime, China). The protein samples were mixed with sample loading buffer followed by boiling for 10 min, and subjected to SDS-PAGE before transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with primary antibodies (Supplementary Table 1) and HRP-conjugated secondary antibodies. Western Lighting Chemiluminescence Reagent Plus (Thermo Fisher Scientific, Inc.) and an Image Quant LAS 4000 biomolecular imager were used to visualize the expression levels.

Human Apoptosis Array

To evaluate the relative expressions of 35 apoptosis-related proteins, we used the Human Apoptosis Array Kit (R&D Systems, Minneapolis, MN, USA). Briefly, nitrocellulose membranes were blocked with an array buffer for 1 h at RT. Protein lysates were diluted and incubated overnight. After washed with 1× wash buffer to remove unbound proteins, membranes were exposed to a cocktail of biotinylated detection antibodies for 1 h at RT. Membranes were washed and incubated with streptavidin-HRP for 30 min at RT. Each capture spot corresponding to the amount of apoptotic bound protein was detected with enhanced chemiluminescence western blotting luminol reagent and visualized by X-ray film.

Statistical analysis

All experiments were performed at least three times. All data are presented as the mean ± standard deviation. Statistical analyses were conducted with Microsoft Excel 2010 Professional Plus (Version 14.0.7237.5000) and GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, CA). Differences between two groups with similar variance were compared using Student's t-tests. For all tests, a $p<0.05$ was considered to indicate a statistically significant difference.

Results

DHA inhibits the proliferation of SGC-7901 cells in vitro

To test the effects of DHA (Fig. 1A) on cancer cell viability, we performed a CCK-8 assay to detect cell viability with DHA treatment in gastric cancer SGC-7901. The results revealed that the viability of SGC-7901 was inhibited in a dose-dependent manner by DHA treatment for 48 h (Fig. 1B). The IC_{50} of SGC-

7901 cells was about 4 mg/ mL. Besides, we conducted clone formation experiments, and the results showed that DHA induced inhibition of the Colony formation assays of SGC-7901 cells, and the plates with fewer cells could be seen from 2 μg/ml to 4 μg/ml with the increase of doses (Fig.1C), which was consistent with the cell viability results.

DHA induces apoptosis in SGC-7901 cells

To explore the effects of DHA on apoptosis, we treated SGC-7901 cells with DHA at concentrations of 0, 5 and 10 mg/ml for 48h, and stained them with Annexin V and PI, followed by flow cytometry. The results showed treatment with 5, and 10 mg/ml of DHA significantly increased the proportion of apoptotic cells in a dose-dependent pattern (Fig. 1D). The apoptosis was further observed in the Hoechst staining assay, DHA treatment caused significantly decreased numbers of cells. However, apparent higher percentages of cells with a more prominently stained nucleus were observed in the microscope field of view, suggesting that apoptosis was triggered in these cells (Fig. 1E). To determine how DHA induced apoptosis in SGC-7901 cells, we used the human apoptosis array to assess the relative expression levels of 35 apoptosis-related proteins (Fig. 2A). Compared with the control group, expressions of caspase-3, HSP27, and HSP70 were markedly reduced while the protein levels of cleaved caspase-3 and cytochrome c increased in a dose-dependent manner (Fig. 2B).

We used qPCR to measure the differential expression of conventional apoptosis-related markers (Fig. 2C). The mRNA level of Bcl-2 was significantly decreased while the level of Bax was significantly increased in a dose-dependent manner. In the western blot assay, increased Bax and Bim were observed. The levels of Caspase 9, Bcl-2 were decreased. The protein level of PARP was suppressed, while correspondingly, C-PARP, which was absent in the control and 5 mg/ml DHA group, was upregulated in the 10 mg/ml DHA group.

DHA induces ER stress, and an inhibitor of ER stress neutralizes the DHA-induced Cell death

Several sensors of ER stress were measured at mRNA and protein levels. Treatment of SGC-7901 cells induced enhanced expression of GRP78, calnexin, calreticulin, ATF4 and CHOP at the mRNA level (Fig. 3A). The ER stress pathway-associated proteins were markedly altered at the protein level as shown in western blot analysis. The levels of ATF4, CHOP, GRP78, and IRE1 increased in a dose-dependent manner (Fig. 3B). The expression of PERK and c-Jun remained unchanged. However, DHA treatment increased the level of phosphorylated c-Jun level compared with the control group. To investigate whether ER stress was required for the DHA-induced apoptosis, we used ER stress inhibitors to block the ER stress pathway. KIRA6, an IRE1 inhibitor, reversed the apoptosis. KIRA6 could significantly reverse the apoptosis of SGC-7901 cells induced by DHA in three repeated experiments (Fig. 4A). As expected, the expression level of p-c-Jun decreased while that of Bcl-2 increased with increasing concentrations of the inhibitor (Fig. 4B). Consistent with the cell viability and western blot results, the Hoechst staining analysis also showed that less severe DNA damage was found in the KIRA6-treated groups (Fig. 4C). All these results implied that the DHA-induced cell death was through the ER stress pathway.

Discussion

Consistent with findings in other reports, our study found that DHA inhibited the proliferation of SGC-7901 cells in CCK-8 assay in a dose-dependent manner. This anti-proliferative effect was also observed in the colony formation assays.

Apoptosis is a type of programmed death by which tissues remove redundant or damaged cells to secure homeostasis [15]. Apoptosis is initiated when the anti-apoptotic molecules are blocked by pro-apoptotic ones. Bcl-2 plays a critical role in the mitochondrial apoptosome-mediated intrinsic pathway of apoptosis [16]. Bax, upon apoptotic signals, binds to Bcl-2 directly to inhibit the latter's anti-apoptotic effect. The Bax/Bcl-2 complex stimulates the release of cytochrome c, and later on, activates the caspase pathway, which at last triggers apoptosis in the cells [17]. In our experiments, the apoptotic effect of DHA was first observed in the annexin V-FITC and PI staining assay in flow cytometry analysis. In the Hoechst staining assay, DHA treatment caused apparent nuclear condensation, indicating that the cells were undergoing apoptosis. The apoptotic effects were further validated in the Apoptosis Array Kit assay. The expression of Bcl-2 was suppressed while the expression of Bax, cytochrome c, and cleaved caspase-3 was markedly upregulated. It is worthy to be mentioned is that two heat shock proteins, HSP27 and HSP70, were markedly decreased after DHA treatment. Heat shock proteins are a family of molecular chaperones involved in protein folding and are induced by heat shock or other stressors [18]. Both HSP27 and HSP70 play significant roles in cancer development and metastasis by inhibition of apoptosis [19, 20]. The reduction of HSP27 and HSP70 further implies that DHA triggers apoptosis by blocking the anti-apoptotic targets.

We continued to investigate the impact of other biological processes, e.g. ER stress, on the lethality of DHA toward SGC-7901 cells. After DHA treatment, although the PERK level remained unchanged, we observed enhanced expression of GRP78, IRE1α, ATF4, ATF6, and CHOP in the SGC-7901 cells, indicating that ER stress pathway was activated. ER stress occurs when the protein folding capacity of the Endoplasmic Reticulum is disturbed by hostile micromilieu such as high metabolic demand and oxidative stress in tumor cells [21]. ER stress has been observed in many types of tumor cells treated by DHA, ranging from lung adenocarcinoma cells to colorectal cancer cells. Unfolded protein response, which recovers ER homeostasis and promotes cell survival, is the major pathway of ER stress. Its downstream sensors include GRP78, PERK, ATF6, IRE1α, ATF4, and CHOP. The unfolded protein response splits into several subpaths. One is through the PERK/Eif2α/ATF4 subpath and another one is the IRE1/c-Jun subpath. Both subpaths play a role in regulating cell growth and survival under hypoxic stress [22]. PERK stimulates the phosphorylation of Eif2α, which activates the transcription factor ATF4 to bind to the target pro-survival genes that are involved in oxidative stress, protein folding, and differentiation [12]. Activated IRE1 recruits the molecule TRAF2 and ASK1, leading to activation of the downstream c-Jun signaling which correlates with cell death because of the activation of pro-apoptotic protein Bim and suppression of Bcl-2 [23]. As hypoxia is a common feature of cancer, the ER stress pathway is, therefore, a promising target in cancer therapy. In our study, the DHA treatment seemed to mainly act on the ERS stress through the IRE1/c-Jun pathway instead of the most classic PERK/Eif2α/ATF4 pathway as the

inhibitor of the former pathway significantly reversed the DHA-induced cell death while the inhibitor of the latter one only slightly offset the tendency (supplementary Fig. 1). Inhibition of the ER stress pathway downregulated the p-c-Jun but upregulated Bcl-2, indicating DHA acted through ER stress to trigger apoptosis. Interestingly, however, the requirement of ER stress for the DHA-induced cell death seems to vary in different cancer cells. Some studies report negative effects of ER stress on cancer cell death. In glioma cells, inhibition of the PERK-ATF4-HSPA5-GPX4 pathway using siRNA or small molecules increased DHA's lethality [24]. Chen et al. noticed that silencing of ATF4 or CHOP enhanced the sensitivity of tumor cells to DHA-driven apoptosis in HeLa, HCT116, HepG2, and SKOV3 cells [25]. In contrast, some groups noticed that ER stress contributed to DHA-induced cell death. Elhassanny Et al. reported that a dimer of DHA induced ER stress-related apoptosis in colorectal cancer cells [26]. The apoptosis was suppressed by GSK2606414 and salubrinal, two inhibitors of ER stress, suggesting that ER stress is required for the DHA dimer-mediated apoptosis. Our findings agree with the positive contribution of ER stress to DHA's toxicity. What causes these tissue-specific responses to DHA merits further investigation.

Therefore, we propose a mechanism for DHA's toxic effects on gastric cancer cells (Fig. 4D). First, DHA induces the IRE1/c-Jun ER stress pathway by stimulating the expression of GRP, IRE1, and p-c-Jun, etc. The p-c-Jun then decreases the rate of Bcl-2/Bax, which triggers the Caspase apoptosis pathway. Therefore, an inhibitor of IRE1 is able to reverse the apoptotic effect of DHA.

Conclusions

As a whole, DHA treatment inhibited the proliferative activity of gastric cancer cell line 7901 and induced apoptosis. The cell death was triggered through the ER stress pathway which could be offset by an ER stress pathway inhibitor. We hope that our understanding of the mechanism of DHA-induced antitumor activity would provide new insight into the application of DHA in the treatment of gastric cancer.

Abbreviations

ATF4: activating transcription factor 4;ATF6: activating transcription factor 6;Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma 2; Bim: Bcl-2-interacting mediator of cell death; CCK-8: cell counting kit-8; CHOP: C/EBP homologous protein; DHA: dihydroarteminsin; ER: endoplasmic reticulum; GRP78: sglucose-regulated protein 78/Binding immunoglobulin protein; HSP27: heat shock protein 27; HSP70 heat shock protein 70; IRE1: inositol-requiring enzyme 1; PARP: poly(ADP-ribose) polymerase; PERK: pancreatic ER kinase (PKR)-like ER kinase

Declarations

• Ethics approval and consent to participate

This manuscript does not involve any animal experiment, human participants, human data or human tissue.

• Consent to publish

All the authors agree to publish the data in the manuscript.

• Availability of data and materials

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

• Competing interests

The authors declare that they have no competing interests.

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• Authors' Contributions

Si Chen and Hanlin Wang performed the experiments. Jian Zhang and Lixin Xia conceived and designed the study. Tie Chen, Furong Deng and Hongbin Li assisted the experiments and data analysis. Jian Zhang and Lixin Xia wrote the manuscript. All authors read and approved the manuscript.

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Figures

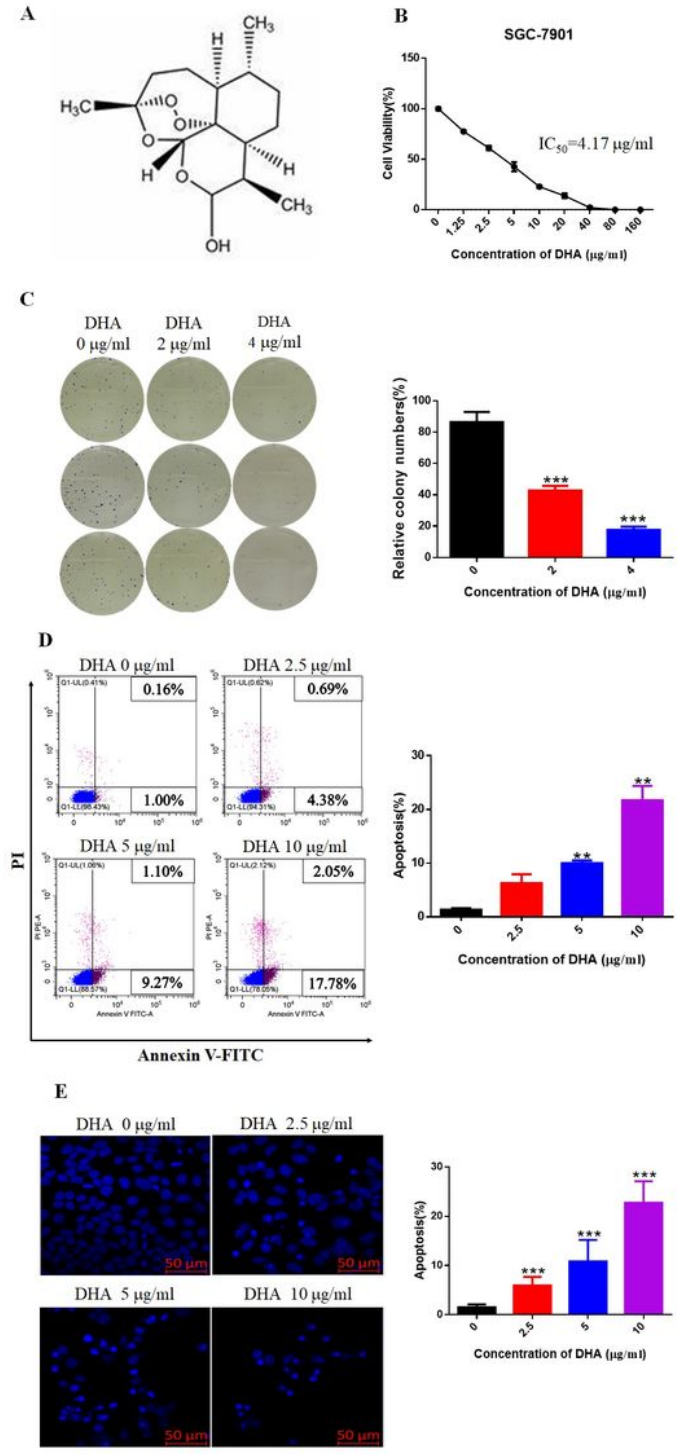

Figure 1

DHA inhibits cell viability, colony forming and induces apoptosis in SGC-7901 cells. A. The molecular structure of DHA. B. SGC-7901 cells were treated with DHA for 48 h and cell growth was examined by CCK8 assays. C. The inhibitory effect of DHA was demonstrated by colony assay in SGC-7901 cells. Colony numbers were quantified from colony assay in DHA-treated SGC-7901 cells. D. Apoptosis was induced in SGC-7901 cells by DHA treatment for 48 h. Annexin V and PI staining were used to label early-

and late-apoptotic cells, respectively. E. Characteristic apoptotic cells were treated with 5 μg/ml and 10 μg/ml DHA for 48 hours. Scale bar = 50 μm. The experiments were repeated twice, with similar results. **p < 0.01, ***p < 0.001.

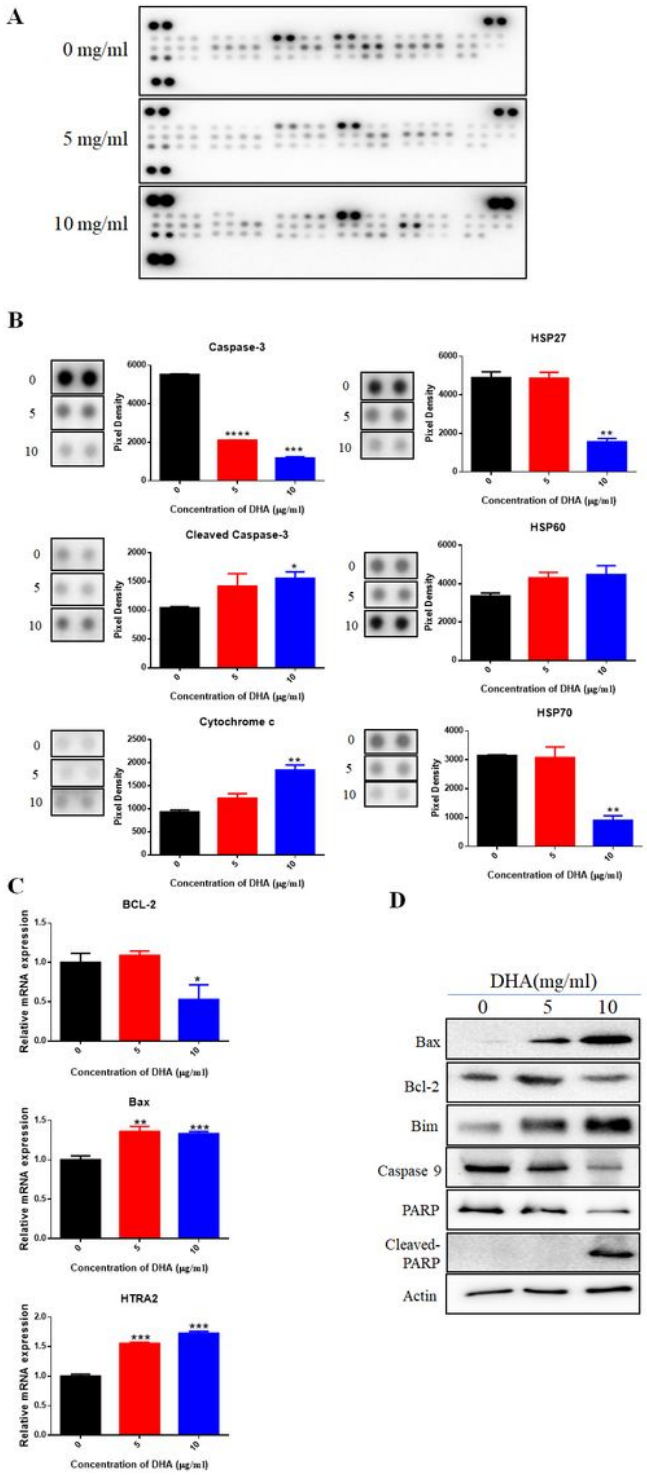

Figure 2

Target profiling SGC-7901 cells treated with DHA. SCG-7901 cells were treated with DMSO or (5,10) μg/ml concentration of DHA for 48 h. A. Entire images of the human apoptosis array. B. Target verification of

results from the human apoptosis array by western blotting. Actin was used as a loading control. The differences in expression levels of four proteins were analyzed using Image J software. C. SGC-7901 cells were treated with DHA for 48 h, and the expression levels of apoptosis-related proteins were detected by qPCR. D. The expression of apoptosis-associated molecules was detected by western blot following 0, 5, 10 μg/ml DHA treatment for 48 h *p < 0.05, **p < 0.01, ***p < 0.001.

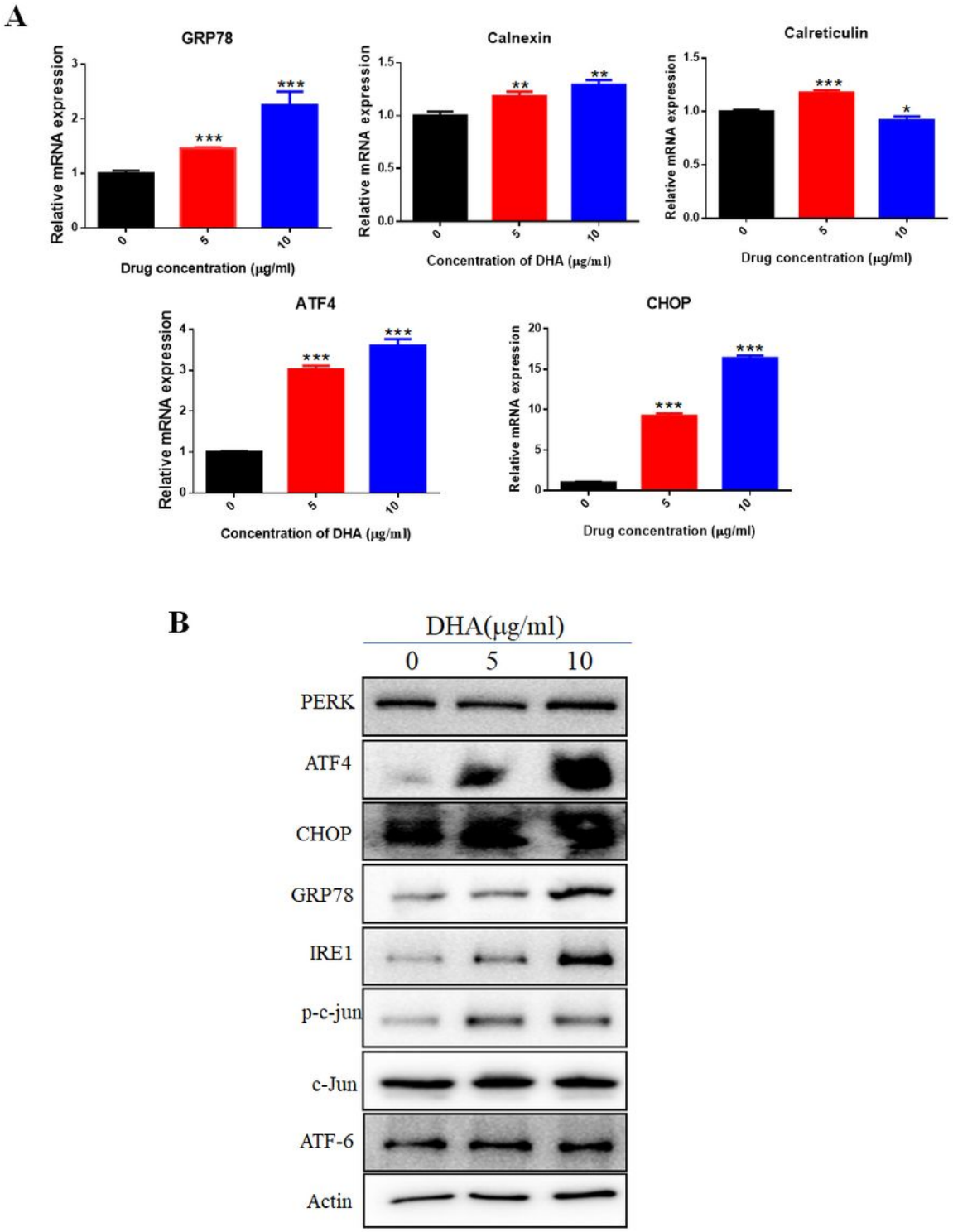

Figure 3

DHA treatment activates apoptosis through the ER stress signaling pathway. A. SGC-7901 cells were treated with DHA for 48 h, and the expression levels of ER stress-related proteins were detected by qPCR. The data are presented as mean ± standard deviation. B. The expression of ER stress-associated molecules was detected by western blot following 0, 5, 10 μg/ml DHA treatment for 48 h. *p < 0.05, **p < 0.01, ***p < 0.001.

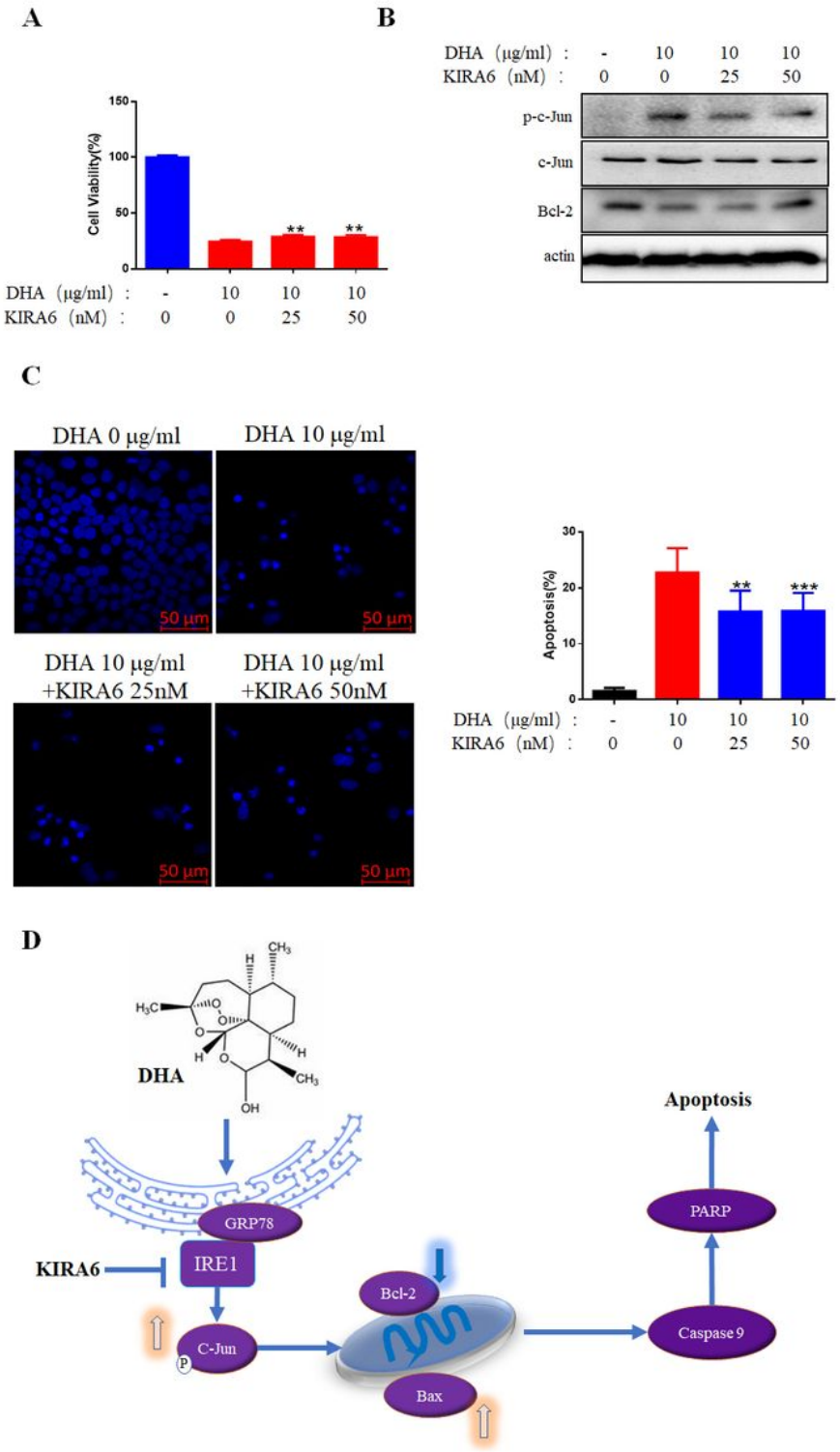

Figure 4

DHA induces ER stress response through the IRE1-c-Jun signaling pathway. A. The cell viability was measured following treatment with KIRA6 and DHA for 48 h in SGC-7901 cells. B. SGC-7901 cells were treated by KIRA6 combined with DHA for 48 h, and the protein expression of ER stress markers, pro-apoptotic markers, and anti-apoptotic markers was determined. C. After treatment of KIRA6 combined with DHA for 48h, the cells were stained with Hoechst staining. The images were observed and photographed under a confocal microscope. Scale bar = 50 μm. The experiments were repeated twice. The data are presented as mean ± standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001. D. Signaling pathway of apoptosis induced by DHA in SGC-7901 cells.

Supplementary Files

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