Dihydroartemisinin (DHA) induces ferroptosis and causes cell cycle arrest in head and neck carcinoma cells

Renyu Lin, Ziheng Zhang, Lingfeng Chen, Yunfang Zhou, Peng Zou, Chen Feng, Li Wang, Guang Liang

Chemical Biology Research Center, School of Pharmaceutical Sciences, Wenzhou Medical University, Wenzhou, Zhejiang 325035, China
Department of Otorhinolaryngology, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang 325035, China
Department of Pharmacy, The Sixth Affiliated Hospital of Wenzhou Medical University, Lishui, Zhejiang 323000, China
School of Environmental and Biological Engineering, Nanjing University of Science and Technology, Nanjing, Jiangsu 210094, China

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide with an incidence of over 500,000 cases each year [1–3]. HNSCC also accounts for approximately 350,000 annual deaths globally [1]. Despite advances in surgery, chemotherapy, and radiotherapy, the five-year survival rate has remained essentially the same. Currently, the treatment for early-stage HNSCC includes either surgery or radiotherapy [4]. At advanced stages, treatment may be multimodal with surgery and chemotherapy [5].

However, high risk of relapse and metastasis following treatment warrants the identification and development of new and efficient treatment options for patients with HNSCC.

Dihydroartemisinin (DHA) is a derivative and active metabolite of the artemisinin (ART). Both were first successfully isolated from Chinese medicinal herb, Artemisia annua. DHA is one of the first-line antimalarial therapies recommended by the World Health Organization. The anti-malarial mechanism of these endoperoxide-containing sesquiterpenes involves reaction with Fe²⁺ ions to generate radicals. In addition to potent anti-malarial activity, DHA has been shown to possess antiviral and antibacterial activities [6,7]. Since ARTs have an established safety record for the treatment of malaria, there has been considerable interest in re-purposing this class of compounds for other indications such as cancer. Numerous large scale epidemiological and pharmacological studies have also shown that ARTs and DHA have efficient and selective anti-tumor activities [8–11]. However, the underlying mechanisms are not fully known.

Antitumor activity of DHA and ARTs has been attributed to apoptosis induction [8,12–14], cell cycle arrest [12,15,16], reduced cell proliferation [13,14,16,17], alteration of tumor-associated genes [18], and inhibition of tumor angiogenesis [19,20]. In HNSCC, studies evaluating the effect of DHA and possible underlying mechanisms...
are scarce. Just recently, DHA was shown to strongly inhibit cell proliferation in CNE-2 HNSCC cell line [21]. Reduced cell numbers together with cell cycle arrest was attributed to apoptosis although this was not directly tested. Similarly, Jia and colleagues showed that DHA inhibited STAT3 activation and inhibited HNSCC growth possibly through induction of apoptosis [22].

Insight into how DHA may specifically target tumor cells came from two recent studies. Oko and colleagues showed that mRNA expression of iron-related genes correlated with the response to cell cycle arrest and induced iron-dependent cell death.

Boosting cancer cell on iron, we hypothesize that DHA causes HNSCC apoptosis. Based on the known mode of action of DHA and dependent cellular processes such as DNA synthesis or may directly lead to cell death by utilizing iron in a specific form of programmed cell death called ferroptosis. This form of cell death is defined by the iron-dependent accumulation of lipid reactive oxygen species and depletion of plasma membrane polyunsaturated fatty acids. The metal ion can cause cleavage of DHA and ARTs endoperoxide bridge via the Fenton reaction, which leads to the generation of reactive oxygen species (ROS).

In the present study, we have utilized a panel of HNSCC lines to understand the mechanisms underlying the anticancer activity of DHA. Based on the known mode of action of DHA and dependence of cancer cell on iron, we hypothesize that DHA causes HNSCC cell death by inducing ferroptosis. Here we show that DHA does specifically target HNSCC cells and reduces viability. DHA also caused cell cycle arrest and induced iron-dependent cell death.

Materials and methods

Cell lines and reagents

Human larynx carcinoma HEP-2, nasopharyngeal carcinoma cell lines (5-BF, CNE-1, CNE-2, CNE-2Z), non-cancerous immortalized nasopharyngeal epithelial cell line NP-69, human hepatocyte cell line HL-7702, and human umbilical vein endothelial cells (HUVECs) were purchased from the Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). HUVECs were maintained in DMEM medium. Other cell lines were cultured in RPMI 1640 medium in (Gibco, Thermo Fisher) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, Utah), 0.2% of P/S antibiotic (100 U/mL penicillin, 1 mg/mL streptomycin; Gibco). Dihydroartemisinin (DHA) and Deferoxamine (DFO) were purchased from Aladdin Bio-chem Tech Co. LTD (Shanghai, China); MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). FITC Annexin V Apoptosis Detection Kit I, propidium iodide (PI) and Matrigel Basement Membrane Matrix were purchased from BD PharMingen (Franklin Lakes, NJ). Antibodies including anti-CD26, anti-MDM2, anti-β-c1, anti-FOXO1, anti-BAD, anti-CyclinB1, anti-p53 (including phospho-p53), anti-GADD, anti-mouse IgG-HRP, donkey anti-rabbit IgG-HRP and donkey anti-goat IgG-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against E-RAS and HIF-1α were purchased from Cell Signaling Technology (Danvers, MA). Caspase-3 activity kit, ROS detection kit, and Hoechst33258 were obtained from Beyotime Institute of Biotechnology (Shanghai, China). Caspase3 inhibitor Z-VAD-FMK was purchased from Santa Cruz Biotechnology. MMP-9 and VEGF ELISA kits were purchased from Westang Biotech Co. LTD (Shanghai, China). FOXM1 siRNA (5′-GGACCAUCUUUCUCCAUUdTdTr-3′) was purchased from GenePharma (Shanghai, China).

Cell viability analysis

Cell viability was evaluated by MTT assay. Briefly, cells were seeded at a density of 2500 cells per well in 96-well plates and incubated overnight in RPMI-1640 medium with 10% FBS in a final volume of 100 μL. The following day, cells were treated with increasing concentrations of DHA (4.4, 8.75, 17.5, 35, 70, and 140 μM) or treated with DMSO alone as control for 24 h, 48 h or 72 h. A total of 20 μL of MTT (5 mg/mL) was added to each well and cells were incubated for 4 h. Then, the medium was discarded, and 150 μL of DMSO was added to each well. Absorbance was measured at 495 nm using a microplate reader (Biotek Instruments, Inc., USA). Cell viability was expressed as a percentage of control cells and the concentration of drug required to obtain 50% inhibition in cell viability was determined as IC50 (also known as growth inhibitory concentration 50; GIC50). IC50 values were calculated by GraphPad Pro Prism 5.0 (GraphPad, San Diego, CA).

Cell cycle analysis

Cell-cycle distribution was determined by cytometric analysis. HEP-2, 5-BF, CNE-1, CNE-2, and CNE-2Z cells were seeded at a density of 300,000 cells per well of 6-well plates and cultured overnight. Cells were then treated with increasing concentrations of DHA (7.5, 35, 70, and 140 μM) for 24 h. For some experiments, cells were transfected with siRNA targeting FOXM1 using Lipofectamine 2000 for 24 h and cultured for another 48 h prior to DHA exposure. To determine the involvement of ferroptosis, cells were treated with deferoxamine (DFO), a high-affinity Fe (III) chelator, at 100 μM alone or in combination with 140 μM DHA for 24 h. After treatment, cells were harvested, washed twice with ice-cold PBS, and fixed with 70% ethanol for at least 2 h at −20°C. Then, cells were pelleted, resuspended in PI staining solution, and incubated at room temperature in the dark for 10 min. Flow cytometric analysis of cell cycle was conducted using FACScalibur flow cytometer (BD Biosciences, CA) and results were analyzed using FlowJo software (TreeStar, Ashland, OR).

Western blot analysis

Cells were suspended in a protein lysis buffer on ice. Protein concentration of the samples was determined by Bio-Rad protein assay (Bradford). Samples were separated on precast 8%–15% SDS polyacrylamide gels and proteins transferred to PVDF membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 90 min at room temperature. Incubations with primary antibodies were carried out in TBS-T overnight at 4 °C. Horseradish peroxidase (HRP)- conjugated secondary antibodies (1:4000; 1 h) and enhanced chemiluminescence detection system (ECL, GE Healthcare) were used for protein measurement. GAPDH was used as the loading control.

Enzyme-linked immunosorbent (ELISA) assay

HEP-2 and CNE-1 cells were seeded at a density of 300,000 cells per well in 6-well plates and cultured overnight to allow for cell attachment. Cells were then treated with increasing concentrations of DHA (35, 70, and 140 μM) for 24 h. DMSO was used as negative control. Culture media was collected, centrifuged to remove dead cells and debris, and assayed for vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9) levels using ELISA (Westang, Shanghai, China).

HUVEC tube formation assay

Endothelial cell tubule formation was assessed by using condition media produced by HUVEC lines treated with DHA. HEP-2 and CNE-1 cells were seeded at a density of 300,000 cells per well in 6-well plates and cultured overnight as before. Cells were treated with increasing concentrations of DHA (35, 70, and 140 μM) or treated with DMSO alone as negative control for 24 h. Media was aspirated and cells were cultured in serum-free medium for additional 24 h. Media was collected, centrifuged, and stored at −80°C (condition media). Growth factor-reduced Matrigel was added to pre-chilled 96 well plates and the plates were then incubated at 37°C for 30 minutes. HUVECs were trypsinized and seeded at 3000 cells per well of the 96-well plate containing Matrigel. DMEM medium supplemented 1:1 with cell-free condition media was then added (DMEM alone served as control). The plates were then incubated for 6 h. Tubule formation was inspected and scored using an inverted microscope (Nikon, Japan). To determine whether DHA alone (directly) may influence endothelial cell tubule formation, HUVECs seeded on Matrigel were treated with DMSO or increase concentrations of DHA directly (35, 70, and 140 μM). At least three different fields were randomly selected in each well and photographed. Tubule capillary length was measured using Image-Pro Plus 6.0 software. All experiments were performed at least three times.

Determination of cell death

Cell death was determined using Annexin V apoptosis detection kit. After indicated treatments, cells were harvested, re-suspended in 500 μL binding buffer, and stained with 1 μL FITC-conjugated anti-Annexin V for 10 min and then 1 μL Propidium iodide (PI) for 5 min. Apoptosis was analyzed with a FACScalibur flow cytometer and the results were analyzed using FlowJo software. Quantification was performed from data generated from at least 3 individual experiments.

Caspase-3 activity assay

Caspase-3 activity was measured in cell lysates after indicated treatments. Total lysate protein concentration was determined by BCA protein assay. Ten μL of
supernatant was incubated in a 96-well plate with 10 μL Ac-DEVD-pNA (2 mM) for 2 h at 37 °C. The release of p-nitroanilide (pNA) was measured by determining the absorbance at 405 nm using Multiskan Spectrum (Thermo Fisher). Relative caspase-3 activity was calculated as a ratio of absorbance of treated cells to untreated cells. For some experiments, cells were co-treated with 4 μM pan-caspase inhibitor z-Vad-fmk and 140 μM DHA for 24 h before determining caspase-3 activity.

Measurement of reactive oxygen species (ROS)

ROS levels in cells were measured by using dichlorofluorescein diacetate (DCFH-DA). Briefly, 300,000 HEP-2 and CNE-1 cells were plated in 6-well plates and cultured overnight. After indicated treatments, cells were washed with serum-free culture medium and incubated with 5 μM DCFH-DA for 30 min. Cells were then washed and harvested, and fluorescence intensity was quantified by flow cytometry. Results were analyzed using FlowJo software.

Hoechst staining assay

HEP-2 and CNE-1 cells were seeded at a density of 200,000 cells per well in 35 mm plates. Cells were treated with 140 μM DHA or DMSO for 24 h. DFO-treated cells (as outlined above) were also used. Cells were washed and incubated with 10 mg/mL Hoechst staining solution for 5 min at room temperature. Samples were examined under a fluorescent microscope (Nikon, Tokyo, Japan).

Transmission electron microscopy

HEP-2 cells and CNE-1 cells were seeded at a density of 600,000 cells on 60 mm plates. Cells treated with 140 μM DHA, 100 μM DFO, or 100 μM DFO + 140 μM DHA were trypsinized and fixed in 2% glutaraldehyde in 0.1M PBS (pH-7.4). Post-fixing was performed in 1% osmium tetroxide for 1 h at 4 °C. Cells were dehydrated through a graded ethanol series and embedded in epon. Sections were then sliced into semithin sections and stained with toluidine blue. Ultrathin sections (70 nm) were placed on polyvinyl (formvar)-coated slot copper grids and counterstained in uranyl acetate and Reynolds lead citrate. Sections were observed under electron microscope (H-7500, Hitachi, Ibaraki, Japan).

Cell transfections and gene silencing

siRNA targeting FOXM1 were designed (5'-GGACCACUUUCCCUACUUdTdT-3′) and used with Negative Universal Control (NC) (Thermo Fisher). Cells were transfected using Lipofectamine 2000 (Thermo Fisher) for 48 h. After another 48 h culture, FOXM1 expression was verified by western blot.

Statistical analysis

Data represent at least 3 independent experiments and expressed as means ± SEM. All statistical analyses were performed using GraphPad Pro Prism 5.0. One-way ANOVA followed by multiple comparisons test with Bonferroni correction was employed to analyze the differences between sets of data. P value < 0.05 was considered significant.

Results

DHA specifically reduces viability of HNSCC cell lines

We first tested the effect of DHA on cell viability utilizing a panel of head and neck cancer cell lines (5-8F, CNE-1, CNE-2Z, CNE-2, HEP-2) and two normal epithelial cell lines (nasopharyngeal epithelial cell line NP-69 and human liver cell line HL-7702) by MTT assay. As shown in Fig. 1A–C, DHA reduced cell viability in a dose-dependent manner in all five HNSCC cell lines. DHA also exerted its pharmacological effect in a time-dependent manner in cancer cells. Surprisingly, DHA had no effect on the viability of the two non-tumorigenic cell lines NP-69 and HL-7702. Among the HNSCC cell lines profiled, DHA treatment for 72 h displayed similar inhibitory efficacy in HEP-2 cells (IC50 = 18.1 μM) and CNE-1 cells (IC50 = 18.4 μM) (Fig. 1D). The IC50 values shown in the figure denote the concentration needed to achieve 50% response in the viability assay. Based on this data, HEP-2 and CNE-1 cells were used in subsequent experiment to detail the mechanisms underlying reduced viability.

DHA induces cycle arrest in HNSCC cell lines

To better understand whether DHA reduces cell viability by altering cell cycle distribution, we performed cell-cycle analysis by

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**Fig. 1.** Effect of DHA on HNSCC viability. Five HNSCC cell lines and 2 non-tumorigenic cell lines were plated and treated with different concentrations of DHA. Cell viability was assessed by MTT at 24 h (A), 48 h (B), and 72 h (C). X-axis shows DHA concentration in log scale. (D) Calculated IC50 at different time points (50% effect; viability). Data show that DHA reduced cell viability specifically in the HNSCC lines. No effect on viability was seen in normal epithelial lines [5-8F, CNE-1, CNE-2Z, CNE-2, HEP-2 = HNSCC lines; NP-69 and HL-7702 = normal nasopharyngeal and liver epithelial cell lines, respectively].
flow cytometry. As shown in Fig. 2A and B, DHA treatment increased the proportion of cells in the S and G2/M phase in a dose-dependent manner. However, there was no significant alteration in G1 phase population. To confirm these findings, we performed western blot analysis of G2/M phase-related proteins cyclin B1, CDC2, and MDM2. Our data show that DHA decreased cyclin B1, CDC2, and MDM2 levels in both HEP-2 and CNE-1. We also found that FOXM1 was decreased upon DHA exposure in dose-dependent manner and this suppression was evident as early as 6 hours post-DHA treatment (Fig. 2C and D). FOXM1 is known to play a key role in cell cycle progression and regulates the expression of large array of G2/M-specific genes. These results reveal that DHA treatment causes marked cell cycle arrest in HNSCC cells.

DHA inhibits the angiogenic activity of HNSCC cell lines

It is well known that artemisinin-based drugs can inhibit angiogenesis in tumors and reduce tumor growth. Thereafter, we tested whether DHA treatment could also inhibit the angiogenic activity of HNSCC cell lines. For this, we measured VEGF and MMP-9 as surrogate markers of angiogenic activity as a starting point. Both VEGF and MMP-9 are essential for angiogenesis and recent reports show that DHA can inhibit VEGF and MMP-9 in pancreatic cells [26]. In our study, DHA resulted in decreased production of both VEGF and MMP-9 by HEP-2 and CNE-1 cells (Fig. 3A). We next performed an in vitro endothelial cell tubule formation assay to further assess the effect of DHA on angiogenesis. As shown in Fig. 3B, conditioned media from HEP-2 and CNE-1 cells treated with DHA inhibited endothelial tube formation. Quantification of tubule length shows that this effect is dose-dependent (Fig. 3C). This inhibition was not seen when DHA was directly added to the endothelial cell cultures (data not shown), suggesting that DHA alters the HNSCC cells to a phenotype less supportive of angiogenesis.

DHA induces cell death in HNSCC cell lines

Recent studies show that artemisinin and its derivative can induce apoptosis of hepatocellular carcinoma cells by up-regulating the expression of BAX and down-regulating the expression of bcl-2 [27]. Thus, we used Annexin V/propidium iodide (PI) staining to determine the effects of DHA treatment on cell death induction in HNSCC cells. As shown in Fig. 4A and B, DHA induced a dose-dependent increase cell death in all of five HNSCC cell lines after a 24 h. However, these results were interesting as DHA treatment clustered cells in the upper left quadrant (PI positive, Annexin V negative). Western blot analysis showed reduced expression of bcl-2, p53, phosphorylated p53, and increased BAX upon DHA treatment of cells (Fig. 4C and D). Furthermore, cleaved caspase-3 levels as well as caspase-3 activity were induced by DHA at the highest concentration tested. These results suggest induction of apoptosis in HNSCC by DHA at 140 μM. However, findings from Annexin V/PI staining also point to a mechanism of cell death other than apoptosis.

DHA induces ferroptosis in HNSCC cell lines

Artemisinin derivatives have been suggested to cause ferroptosis in tumor cells [23]. Treatment of 60 cancer cell lines with artemisinin...
derivatives altered the expression of iron-related genes [23]. In addition, artesunate was recently shown to induce ferroptosis in pancreatic cancer cells [28]. This raises the exciting possibility that DHA may contribute to HNSCC cell death through ferroptosis. To test this possibility, we first examined whether Fe$^{2+}$ was required for DHA action. As shown in Fig. 5A and B, co-treatment of cells with potent iron-chelator desferrioxamine (DFO) completely abrogated DHA-induced cell death. Moreover, DFO prevented DHA-induced decrease in bcl-2, p53, and phospho-p53. However, BAX did not show any changes (Fig. 5C). These results show that iron is involved in DHA activities and if there is a non-caspase dependent mechanism of cell death, it too depends on intracellular iron.

An important mechanism in ferroptosis-mediated cell death is reactive oxygen species (ROS) production and oxidative stress [29,30].

![Fig. 3. Reduced angiogenic phenotype of HNSCC following DHA treatment.](image-url)
We measured ROS production in both HEP-2 and CNE-1 cells exposed to DHA by using DFCH-DA. DFCH-DA produces fluorescence DCF in the presence of intracellular ROS. Our results show that DHA increases ROS levels in a dose-dependent manner (Fig. 5C). This activity was completely attenuated in the presence of DFO (Fig. 5D).

We then measured surrogate markers of ferroptosis GPx4 and Ras in cells exposed to DHA. Studies have shown that inactivation of GPx4 induces ferroptosis [31]. Furthermore, Ras has been shown to protect against oxidative stress-induced ferroptotic cell death [32]. Our results show that DHA decreases the levels of both glutathione peroxidase 4 (GPx4) and Ras in HNSCC cells (Fig. 6A). Since both GPx4 and Ras are negative regulators of apoptosis, a decrease in the levels is indicative of ferroptotic cell death in HEP-2 and CNE-1 cells. Furthermore, DHA-induced decrease in GPx4 and Ras is inhibited by DFO (Fig. 6B). This prevention of GPx4 and Ras reduction coincides with restoration of Bcl2, Bax, p53, and cleaved caspase-3 levels by DFO (Fig. 6B). Collectively, our data show that iron is essential for DHA-mediated cell death and may represent a link between apoptosis and ferroptosis in HNSCC cells. In support of this notion, we show that DHA induces caspase-3 activity and this induction is completely prevented in the presence of DFO (Fig. 6C). As expected from these results, co-treatment of cells with DHA and pan-caspase inhibitor z-Vad-fmk also showed prevention of DHA-induced caspase-3 activity (Fig. 6C).

DHA-induced cell cycle arrest is dependent on ferrous ion in HNSCC cell lines

Our studies showed that DHA could arrest cells in the S and G2/M phase by modulating the expression of cell cycle regulating proteins. We wanted to know if this arrest also involves iron and could be prevented in the presence of an iron chelator. To test this, we treated HNSCCs with DHA and DFO and show that DFO prevented DHA-induced cell cycle arrest (Fig. 7A and B). This was confirmed by western blot analysis showing maintained expression of FOXM1, cyclin B1, CDC2 and MDM2 in cells co-treated with DHA and DFO.
These data show that DHA causes cell cycle arrest in HNSCC through the involvement of Fe^{2+}.

**Discussion**

Novel therapeutic agents that specifically and efficiently target cancer cells are crucial for developing promising treatment modalities for HNSCC. DHA is an effective treatment drug for falciparum malaria; it shows remarkable safety, is well tolerated, and is widely used in the clinic. Studies are now emerging that show DHA also exhibits strong antitumor activity in different human cancer cells. However, less than a handful of studies have examined the effect of DHA on HNSCC. Inhibition of tumor cell growth by DHA is somewhat established but the mechanisms are still being uncovered. In the present study, we have demonstrated that DHA possesses antitumor properties against a series of human HNSCC cells. We also show that DHA causes cell arrest in a Fe-dependent manner and possibly through modulating FOXM1 levels.

**FOXM1 had no influence on cell death in HNSCC cell lines**

It has been suggested that suppressing the expression of FOXM1 may make cells susceptible to drug-induced cell death. We silenced FOXM1 by siRNA to determine whether DHA induces ferroptosis by decreasing the expression of FOXM1 (Fig. 8A). FOXM1 knockdown decreased the expression of cyclin B1, CDC2, and MDM2 (Fig. 8A), and caused accumulation of cells in the G2/M phase as expected (Fig. 8B and C) but did not induce cell death (Fig. 8D and E). Combination of FOXM1 knockdown and DHA treatment showed a greater effect on increasing G2/M cell cycle arrest compared to FOXM1 knockdown or DHA treatment alone (Fig. 8B and C). No significant changes were noted in cell death number when cells transfected with FOXM1 siRNA were treated with DHA compared to control siRNA transfected cells (Fig. 8D and E). These data show that FOXM1 may participate in DHA-induced cell cycle arrest but not cell death.
cells. Our studies may have also uncovered the mechanisms of tumor cell specificity of DHA.

To date, most studies have reported that ARTs and DHA induce apoptosis in cancer cells. For example, Lu and colleagues found that DHA caused translocation of ROS-dependent BAX, depolarization of mitochondrial membrane, morphological changes in mitochondria, release of cytochrome c and activation of caspase-3 [36]. Beekman et al. also found that ARTs reverse bcl-2/BAX ratio and induce apoptosis [39]. Furthermore, DHA induced a loss of the mitochondrial transmembrane potential ($\Delta\psi_m$), release of cytochrome c, activation of caspases, and externalization of phosphatidylserine indicative of apoptosis induction [27]. We also noted similar changes in bcl-2/BAX ratio as well as mitochondrial changes. We show that DHA also induces caspase-3 activity indicating induction of

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**Fig. 6.** Induction of ferroptotic cell death by DHA. (A) Western blot analysis of glutathione peroxidase 4 (GPx4) and Ras (E-Ras) in HEP-2 and CNE-1 cells following treatment with DHA at different concentrations for 24 h. (B) Analysis of cell death related proteins in HNSCC cells treated with DHA, DFO, or combination of DHA and DFO. C. Caspase-3 activity in HNSCC treated with DHA with or without DFO and with DHA with or without caspase inhibitor z-vad-fmk. (D) Morphological changes in HEP-2 and CNE-1 cells following DHA treatment. Upper panels show phase contrast images; middle panels showing Hoechst staining; lower panels are TEM images (arrows point to mitochondrial structural changes). [DMSO = control; DHA = 140 μM; DFO = 100 μM; DFO + DHA = 100 μM DFO + 140 μM DHA; *P < 0.05, **P < 0.01, ***P < 0.001].
caspase-dependent apoptosis in HNSCC. However, our flow cytometric analysis of cell death was surprising and pointed to another mode of cell death.

Antimalarial mechanisms of ARTs and its derivatives are Fe\(^{2+}\)-dependent. Fe\(^{2+}\) causes the cleavage of endoperoxide bridge in ARTs and derivatives and leads to the generation of ROS. Our results show increased ROS levels in cells treated with DHA. This increase was prevented by an iron chelator DFO. Increased DHA-induced ROS may also be behind the antitumor activity of DHA. In our studies, cell death induced by DHA was prevented when the cells were treated DFO. These results show that DHA requires iron to induce cell death in HNSCCs. Iron is needed for DNA synthesis and tumor cells are heavily dependent on iron for their rapid growth. If tumor cells contain greater amounts of iron than their normal counterparts, it is possible that DHA exploits high iron levels to specifically target cancer cells. We noted that DHA reduced the levels of GPx4 and Ras indicating ferroptotic cell death. This was confirmed by morphological changes reminiscent of ferroptosis seen in tumor cells.

Cell cycle arrest has been suggested to be a key to the antitumor activity of artemisinin derivatives. Steinbruck and colleagues found that artesunate had an anti-mitogenic effect in Saccharomyces cerevisiae and cancer cells. This effect was mediated by arresting cells in the G2/M phase [40]. Hosoya et al. showed that DHA caused cell cycle arrest in G0/G1 phases to inhibit canine osteosarcoma cells growth [41], while Jiao et al. observed G2 phase arrest in ovarian cancer [11]. And lastly, nasopharyngeal carcinoma CNE-2 was arrested in G0/G1 by DHA [21]. Our studies show that DHA treatment arrested HNSCC cells including CNE-2 in the S and G2/M phases. The potential difference between our study and that by Huang and colleagues [21] may be the experimental setup. One important point of distinction is the plating density. Huang et al. used 100,000 cells/cm\(^2\) which may be high for cell cycle analysis. All of our studies were conducted with consistent densities for all assays. We also analyzed G2/M-associated proteins and show that cyclin B1, CDC2 and MDM2 are all decreased upon treatment with DHA. Finally, we measured the expression of FOXM1, a regulator of cell cycle progression.

**Fig. 7.** Effect of DFO on DHA-induced cell cycle arrest. HEP-2 and CNE-1 cells were treated with DFO and DHA to assess the role of iron in DHA-induced cell cycle arrest. (A) Flow cytometric analysis of PI stained cells showing that DFO prevents G2/M phase arrest induced by DHA. (B) Quantification of cells in G2/M phase [bar graphs produced from 3 independent experiments]. (C) Western blot analysis of FOXM1, cyclin B1, CDC2, and MDM2 expression in HEP-2 and CNE-1 cells co-treated with DFO and DHA [*P < 0.05, **P < 0.01, ***P < 0.001].
and show that FOXM1 expression is also suppressed by DHA. siRNA-mediated knockdown of FOXM1 recapitulated the cell cycle arrest in HNSCC but did not induce cell death. A growing body of evidence has implicated a role for FOXM1 in a number of malignancies including breast, liver, lung, prostate, and colorectal cancer [42–45].

FOXM1 typically peaks at G1/S and G2/M phases and is essential for cell division through pushing M phase entry [46,47]. These findings suggest that DHA causes cell cycle arrest possibly by suppressing FOXM1 but induces cell death independent of FOXM1.

A feature common to all solid tumors including HNSCC is that rapid growth of the tumors accompanies angiogenesis. DHA has been shown to inhibit extracellular signal regulated kinase/ERK signaling in endothelial cells [8]. ERK, part of the MAPK signaling family, is well established in cellular proliferation and angiogenesis [48,49]. Furthermore, DHA inhibits VEGF and MMP-9 in pancreatic cells [26]. Therefore, some of the antitumor activities of DHA may be mediated by inhibiting angiogenesis [19]. To determine whether DHA causes alteration of angiogenic phenotype of tumor cells, we assayed for VEGF and MMP-9 production. Our studies show that DHA reduces VEGF and MMP-9 production in HEP-2 and CNE-1 cells in a dose-dependent manner. Condition media from DHA-treated HNSCCs also prevented endothelial cell tubule formation. VEGF expression has been suggested to be an important prognostic factor in head and neck cancer [50] and DHA-mediated decreased VEGF production as shown in our study is remarkable. Our findings demonstrate that DHA pushes HNSCC cells toward a less angiogenesis-supportive phenotype.

In summary, our study showed that DHA exhibits antitumor activity in HNSCC cells by cell cycle arrest, inducing ferroptosis and apoptosis, and inhibiting angiogenesis-supportive phenotype. Identification of ferroptosis and the signaling mechanisms may lead to the development of new therapeutic approaches to regulate tumor growth in HNSCC. The study also highlights DHA as a potential therapeutic drug for HNSCC.

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Conflict of interest

The authors disclose no potential conflicts of interest.

References