MAG-DHA Induces Apoptosis and Autophagy in Breast Cancer Cells via Lipid Peroxidation-Mediated Endoplasmic Reticulum Stress

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Research

Keywords: MAG-DHA, breast cancer, apoptosis, autophagy, lipid peroxidation, endoplasmic reticulum stress

DOI: https://doi.org/10.21203/rs.3.rs-35529/v1

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Abstract

**Background:** Epidemiologic and pre-clinical studies have shown that marine n-3 polyunsaturated fatty acids (n-3 PUFAs) elicit promising chemoprevention against breast cancer. Previous studies found that docosahexaenoic acid monoglyceride (MAG-DHA) does not require pancreatic lipase to be absorbed, unlike DHA-triglyceride which needs to be hydrolyzed by sn-1,3' specific gastric and (colipase-dependent) pancreatic lipases as free fatty acids and monoglycerol prior to intestinal absorption. Therefore, this property confers increased absorption, and thus a better bioavailability when compared with other formulations such as DHA-free fatty acid, DHA-triglycerol (TAG-DHA), or DHA-ethyl ester (EE-DHA). However, the anti-cancer actions of n-3 PUFA monoglyceride on breast cancer remain to be assessed.

**Methods:** SKBR3 and E0771 cells were exposed in vitro to MAG-DHA. Cell viability (by MTT), malondialdehyde (MDA) levels, cell apoptosis and autophagy (by western blot), Beclin1 knockout (by siRNA) was examined. Transmission electron microscopy (TEM) was used for analyzing cell apoptosis and autophagy in vivo breast cancer exnografts.

**Results:** In this study, we showed that docosahexaenoic acid monoglyceride (MAG-DHA) caused oxidative stress as evidenced by MDA accumulation, which triggered endoplasmic reticulum (ER) stress and subsequently induced apoptosis in E0771 and SKBR3 breast cancer cells. In particular, MAG-DHA-induced apoptosis is associated with the activation of the PERK-eIF2α pathway and caspase-12. MAG-DHA treatment also strongly suppressed the growth of E0771 murine breast cancer xenografts, by ER-stress-induced cell apoptosis. In addition, we found that MAG-DHA-induced ER stress concomitantly triggered autophagy in these cancer cells, and the induction of autophagy suppressed its ability to induce apoptotic cell death.

**Conclusions:** Together, our data suggested that MAG-DHA combined with autophagy inhibitors may be a useful therapeutic strategy in treating breast cancer.

Introduction

Breast cancer is by far the most frequently diagnosed cancer among women across the global, with over 2 million new cases estimated in 2018. It also ranks as the leading cause of cancer-related mortality in females worldwide, leading to over 500,000 deaths per year worldwide [1]. Despite recent advances in both diagnosis and treatment, drug resistance and the development of deadly metastases of node-negative or positive breast cancer patients continue to represent a significant clinical challenge [2]. Therefore, preventative strategies, novel interventions and therapeutic approaches are urgently needed.

Recently, a large body of literature has indicated that dietary interventions might be able to reduce breast cancer risk [3, 4]. Among the dietary factors, n-3 polyunsaturated fatty acids (n-3 PUFAs), especially docosahexaenoic acid (DHA, 22:6, n-3) and eicosapentaenoic acid (EPA, 20:5, n-3), naturally present in cold-water fish as well as in fish oil supplements have gained attention as possible candidates for breast cancer prevention. To date, n-3 PUFA supplements are present in foods as triacylglycerol (TAG) or in small
proportions as monoacylglycerol, diacylglycerol (DAG), or phospholipid formulation [5]. There is evidence that the influences of the chemical structures on fatty acids bioavailability have been characterized. Studies have shown that MAG-DHA, a DHA sn-1-monoacylglycerol did not required pancreatic lipase to be absorbed, unlike DHA-triglyceride which needs to be hydrolyzed by sn-1,3’ specific gastric and (colipase-dependent) pancreatic lipases as free fatty acids and monoglycerol prior to intestinal absorption. Therefore, this property confers increased absorption, and thus a better bioavailability when compared with other formulations such as DHA-free fatty acid, DHA-triglycerol (TAG-DHA), or DHA-ethyl ester (EE-DHA). Cruz-Hernandez et al. demonstrated that an enriched sn-1(3)-MAG oil was a better carrier for n-3 long-chain PUFAs (LC-PUFAs) compared with TAG from fish oil [6]. It was also found that MAG-DHA induced powerful inflammation resolution in various models of pulmonary diseases [7, 8]. In addition, recent studies have shown that MAG-DHA displayed anti-tumorigenic effects in colorectal cancer and lung cancer [9, 10]. However, the anti-breast cancer activity of MAG-DHA and its underlying mechanisms remain elusive. The specific objective was to evaluate the effect of MAG-DHA on tumor growth and its underlying molecular mechanisms using an in vitro model of breast cancer cell line SKBR3 and E0771 and an in vivo mouse model of E0771 xenograft.

**Materials And Methods**

**Cell culture and cell viability assay**

The murine E0771 and human SKBR3 breast cancer cell lines (American Type Culture Collection, Manassas, VA, USA) were maintained in RPMI 1640 and Dulbecco’s Modified Eagle’s Medium (DMEM) Supplemented with 10% FBS, 2 mmol/L L-glutamine, and penicillin/streptomycin, respectively. For the testing of MAG-DHA (NU-CHEK, USA) on cell viability change, the MTT assay was used. E0771 and SKBR3 cells were treated with MAG-DHA complexes to BSA in a 4:1 (MAG-DHA/BSA) molar ratio at 20, 40, 80 μM or vehicle for 72 h in medium containing 0.5% FBS. MTT reagent (at 5 mg/ml) was added to each well at final concentration 0.5 mg/ml and the plate was incubated at 37°C. After 4 h, supernatant was carefully removed and DMSO (100 μl) was added. The absorbance was read with a microplate reader (Bio-Rad, China) at 450 nm. The relative cell viability was expressed as a percentage of the control well.

**Lipid peroxidation assay**

The levels of lipid peroxidation marker malondialdehyde were measured in cultured E0771 and SKBR3 cells and tumor tissues. The tests were performed according to the manufacturer’s instructions (Biyotime Biotechnology, Jiangsu, China). The optical density of each well was read with a microplate reader (Bio-Rad, China) at 532 nm.

**Tumor xenograft model**

Murine E0771 xenografts were established in 8-week-old female C57/B6 mice. 20 female C57/B6 mice at 8 weeks of age were obtained from Shanghai SLAC Experimental Animal CO., Ltd (Shanghai, China)
and used according to the approved protocol by the Animal Care and Use Committee of the Ningbo University (Ningbo, China). The mice were housed under aseptic conditions in standard cages in temperature- and humidity-controlled conditions with a 12 h light/dark cycle. Cultured murine E0771 cells were collected, washed twice with serum-free DMEM and counted. Each mouse received a subcutaneous injection of $2 \times 10^6$ viable cells suspended in 100 $\mu$l of serum-free RPMI 1640 medium on day zero. All injections were given to anesthetized animals. After the formation of 100 mm$^3$ tumors, mice were randomly assigned into 2 groups, control (untreated) and MAG-DHA-treated. MAG-DHA was administered per os (400 mg/kg) daily. Tumor size was measured with a caliper every 3 days, and calculated according to the following formula: tumor volume = length $\times$ width$^2 \times 0.5$. Mice were euthanized on week 4 and tumor tissues were removed and stored at −80°C until tissue processing.

Transmission electron microscopy (TEM) analysis for cell apoptosis and autophagy

Tumor tissues were fixed in 4% paraformaldehyde and post-fixed 2% osmium tetroxide before embedding in EPON resin for morphological studies. Ultrathin sections (100 nm on 200-mesh grids) embedded in epoxy resin were double stained with uranyl acetate and lead citrate, and observed with Philips CM10 TEM.

Tumor tissue analysis of DHA

DHA levels of tumor tissues were determined by gas chromatography, as described previously [11]. In brief, tumor tissues were homogenized by grinding it in liquid nitrogen. Total lipids of tumor tissues were extracted using the chloroform: methanol mixture (2:1, v/v), dried under a stream of nitrogen gas and then transmethylated by heating in 14% boron trifluoride in methanol at 80°C for 20 min. Fatty acid methyl esters were then extracted in presence of 2 ml isooctane and separated by Shimadzu GC-14C gas chromatograph system (Shimadzu Corporation, Japan) equipped with a flame-ionization detector (FID) and a capillary column (DB-23, Aglient Corporation, USA). Fatty acids peaks were identified by their relative retention times comparing with those of commercial fatty acid methyl ester (NU-CHEK, USA). DHA level was expressed as a relative percentage of total analyzed fatty acids according to its peak area.

Western blot analysis

Tumor tissues were lysed in Triton x-100 lysis buffer (20 mM Tris-HCl, pH7.6, 1 mM EDTA, 140 mM NaCl, 1% NonidetP-40, 1% aprotinin, 1mM phenylmethysulfonylfluoride, and 1 mM sodium vanadate). Lysates were sonicated and centrifuged at 15000 g for 10 minutes at 4 °C. Protein concentrations were determined using bicinechonic acid (BCA) protein assay kit purchased from Biyuntian Biotech (Haimen, China). Supernatants were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in milk and incubated at 4 °C overnight with primary antibodies followed by incubation with secondary antibodies for 1 h at room temperature. Detection was performed by enhanced chemiluminescence. The primary antibodies rabbit anti-NF-E2-related factor 2 (Nrf2), rabbit anti-Heme Oxygenase 1 (HO-1), rabbit anti-protein kinase R-like ER kinase (PERK), rabbit anti-phospho (p)-PERK (Thr980), rabbit anti-phospho (p)-eIF2α (Ser51), rabbit anti-C/EBP homologous protein (CHOP), rabbit
anti-caspase-12, rabbit anti-cleaved caspase-3, rabbit anti-cleaved PARP, rabbit anti-LC3 I/II, rabbit anti-p62, rabbit anti-Beclin1, mouse anti-β-actin and the horseradish peroxidase-linked secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

**Transfection of siRNAs**

To study the role of Beclin 1 in MAG-DHA-induced loss of breast cancer cell viability, SKBR3 and E0771 cells were transfected (X-tremeGENE siRNA Transfection Reagent, Roche) with Beclin1 siRNAs (Thermo Scientific). The efficacy of the siRNA knockdown of Beclin1 protein expression was determined by Western blot analysis.

**Statistical analysis**

The results were presented as the mean ± SEM and statistical differences were evaluated by one-way ANOVA followed by Newman-Keuls test. *P*<0.05 was considered to indicate statistical significance.

**Results**

**MAG-DHA induces lipid peroxidation-mediated endoplasmic reticulum (ER) stress in cultured breast cancer cells**

To determine the anti-cancer activity of MAG-DHA, the murine E0771 and human SKBR3 breast cancer cells were used as the in vitro models. MAG-DHA was found to significantly suppress the viability (based on MTT assay) of E0771 and SKBR3 cells in vitro and compared with SKBR3 cells, E0771 cells were more sensitive to MAG-DHA (Fig. 1A). Notably, the anti-cancer effect of MAG-DHA in both breast cancer cells was significantly counteracted by N-acetyl-L-cysteine (NAC), a well known antioxidant (Fig. 1A). Since the unsaturated nature of MAG-DHA with 6 double bonds renders its structure more prone to free radicals by triggering lipid peroxidation. To investigate the cellular mechanism by which MAG-DHA induced anti-cancer actions in the in vitro cultured cell model, we therefore focused on examining lipid peroxidation as evidenced by the formation of MDA. As shown in Figure. 1B, MAG-DHA treatment significantly increased MDA level in E0771 and SKBR3 cells. Co-treatment of cells with NAC and MAG-DHA markedly suppressed intracellular MDA accumulation. Next, we sought to determine whether MAG-DHA-induced lipid peroxidation could activate the antioxidant defense system. As shown in Figure. 1C, NF-E2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1), two crucial components of cellular oxidative stress response were significantly up-regulated in E0771 and SKBR3 cells treated with MAG-DHA. These results suggest the antioxidant defense system was activated in response to MAG-DHA-mediated lipid peroxidation. In addition, Co-treatment of cells with NAC and MAG-DHA markedly inactivated the antioxidant defense system (Figure.1C). It is widely accepted that oxidative stress is linked with ER stress, we therefore investigated whether MAG-DHA-induced lipid peroxidation led to ER stress. As expected, we found that the protein levels of phosphorylated protein kinase R-like ER kinase (PERK), a major transducer of ER stress and its downstream target eiF2α were significantly increased in E0771 and SKBR3 cells treated with MAG-DHA, although the total amount of PERK did not change (Figure. 1D). Simultaneously, we also
found that C/EBP homologous protein (CHOP), an ER stress-inducible transcription factors associated with cell cycle arrest and apoptosis was significantly up-regulated in E0771 and SKBR3 cells treated with MAG-DHA (Figure. 1D). Co-treatment of cell with NAC markedly reversed lipid peroxidation-induced ER-stress. In addition, the involvement of ER stress in MAG-DHA-induced growth inhibition was further confirmed in both cells co-treated with 4-PBA, an ER stress inhibitor and MAG-DHA. As shown in Figure. 1E, MAG-DHA-induced loss of cell viability was significantly counteracted by 4-PBA in both cells.

MAG-DHA-induced ER stress results in cell apoptosis and autophagy in cultured breast cancer cells

Next, we investigated whether MAG-DHA-induced ER stress triggered apoptosis induction, we evaluated the typical hallmarks of cells undergoing apoptosis in both breast cancer cells treated with MAG-DHA with immunoblotting analyses. We found that caspase-12, a apoptosis protein specifically activated upon ER stress, the active forms (cleaved forms) of caspase-3 and poly (ADP-ribose) polymerase (PARP), two key mediators of apoptosis were significantly increased in both cells treated with MGA-DHA (Figure. 1F). Co-treatment of cells with 4-PBA prevented MAG-DHA-induced apoptosis pathway activation. Recently, interesting functional links have been revealed between ER stress and autophagy. To determine whether MAG-DHA-induced ER stress also activated autophagy, the protein expression of the autophagy markers microtubule associated protein 1 light-chain 3 (LC3) and Beclin1 was measured with immunoblotting analyses. As shown in Figure. 1E, we found that treatment with MAG-DHA significantly enhanced LC3 and Beclin1 protein levels, and suppressed p62 expression in both cells. Co-treatment of cells with 4-PBA prevented MAG-DHA-induced autophagy pathway activation (Figure. 2A). In addition, we also investigated the effect of autophagy induction in MAG-DHA mediated loss of cell viability using Beclin1 specific siRNAs (Figure. 2B) and autophagy inhibitor chloroquine (CQ). It was found that successful knockdown of Beclin1 by siRNAs and pharmacologic inhibition of autophagy by CQ enhanced MAG-DHA-induced growth inhibition (Figure. 2C&D). These results demonstrated that ER stress-triggered autophagy suppressed MAG-DHA-induced apoptotic cell death.

MAG-DHA inhibits the growth of E0771 xenografts

To evaluate the in vivo anti-cancer efficacy of MAG-DHA on breast cancer, we used the growth of murine E0771 breast cancer cell xenografts in C57/BL6 mice as a representative in vivo model. Following the formation of 100 mm³ tumors, the mice were randomly divided into two groups at day 11, namely control and MAG-DHA-treated. Mice received MAG-DHA per os daily at a pharmacological dose of 400 mg/kg. As shown in Fig. 3B, treatment with MAG-DHA significantly suppressed tumor growth and reduced the size of tumor xenografts in MAG-DHA-treated group compared to control group (Fig. 3A).

Support for the in vitro anti-breast cancer mechanisms in the in vivo tumor xenograft model

To determine whether the mechanism of MAG-DHA-induced cell death observed in vitro also can be seen in vivo, we evaluated the typical hallmarks of cells undergoing apoptosis in tumor tissues with immunoblotting analyses. As shown in Fig. 4A, we found that caspase-12, cleaved caspase-3 and cleaved PARP were significantly increased in tumor tissues in MAG-DHA group compared with control
group. We further evaluated the ultrastructure changes of breast cancer cells from tumor tissues with transmission electron microscopy (TEM). As shown in Figure. 4B, chromatin marginalization, one of classical morphological characteristics of apoptosis was observed in tumor tissues from MAG-DHA group compared with control group. In addition, autophagy markers LC3- and Beclin1 protein levels were significantly enhanced and p62 expression was significantly suppressed in MAG-DHA group (Figure. 4C). The induction of autophagy by MAG-DHA treatment was confirmed by TEM analysis (Figure. 4D). Next, Analysis of the total lipids extracted from tumor tissues showed DHA was significantly elevated in MAG-DHA group (Figure. 5A). Also, increased DHA level in tumor tissues from MAG-DHA group resulted in MDA accumulation and activated the antioxidant defense system (Figure. 5B&C). Furthermore, MAG-DHA-induced lipid peroxidation led to ER stress in MAG-DHA group, as evidenced by increased expression of phospo-PERK, phospo-eIF2α and CHOP (Figure. 6). Our data demonstrated that the proposed anticancer mechanisms of MAG-DHA based on in vitro studies are in line with the observations made with the in vivo tumor sample.

Discussion

In the present study, we found that MAG-DHA induced lipid peroxidation-mediated ER stress, which triggered apoptosis and autophagy in cultured E0771 and SKBR3 breast cancer cells as well as in E0771 xenografts. Notably, MAG-DHA-induced autophagy suppressed its ability to induce apoptotic cell death.

In E0771 xenografts, it is of interest to note that DHA level was increased approximately 4.5fold with MAG-DHA intervention. It is known that the enrichment of DHA with high unsaturation in membranes makes cancer cells more sensitive to free radicals because it makes the membrane less rigid and more vulnerable [12]. Therefore, in the present study, the cell death induced by MAG-DHA was largely mediated by increased lipid peroxidation in cancer cells. The crucial role of lipid peroxidation in MAG-DHA-induced death of breast cancer cells was supported by the following observations made in the present study: First, there was an increase in the intracellular MDA accumulation in cultured breast cancer cells and in MAG-DHA group following treatment with MAG-DHA, respectively. In addition, MAG-DHA-induced lipid peroxidation also activated antioxidant defense system as evidenced by increased expression of Nrf2 and HO-1, two crucial components of cellular anti-oxidative stress response [13]. Second, the cell cytotoxicity, increased MDA levels and up-regulated expressions of Nrf2 and HO-1 induced by MAG-DHA in breast cancer cells were effectively reversed by the antioxidant NAC (Figure. 1). In agreement, Jing et al. demonstrated that DHA-induced cell death of breast cancer cells is associated with increased reactive oxygen species, which activates Nrf2/HO-1signaling pathway [14]. Also, van Beelen et al. reported that the hydrolysed algal oil and fish oil showed similar toxicity on colon cancer cells via oxidative stress [15].

Recently, it was found that oxidized lipids triggered ER stress in rat aortic smooth muscle cells [16]. Protein kinase R-like ER kinase (PERK), a three transmembrane protein induced the unfolded protein response upon ER stress. Activated PERK phosphorylates eIF2α, which results in repression of global protein synthesis to restore cell homeostasis. However, elevated levels of phosphorylated eIF2α also lead to increased translation of selected mRNAs including the transcription factor ATF4, which promotes
expression of CHOP, a transcription factor involved in cell apoptosis [17]. In the present study, in MAG-DHA-treated breast cancer cells or in tumor tissues from MGA-DHA group, we found significant increases in PERK and eIF2a phosphorylation level as well as changes in CHOP expression. Furthermore, the use of antioxidant NAC significantly reversed MAG-DHA-induced ER stress in two breast cancer cells, suggesting that MAG-DHA-induced lipid peroxidation results in ER stress in breast cancer cells. In line with our results, Jakobsen et al. found that DHA induced cell cycle arrest by ER stress in human colon cancer SW620 cells [18]. Similarly, Pettersen et al. also reported that DHA triggered oxidative-stress-mediated ER stress in human colon cancer SW620 cells [19]. However, several in vitro and in vivo studies reported that DHA provides neuroprotection against ER stress [20, 21]. This discrepancy may largely be explained by the cell-specific differences in ER stress response. Ultimately, the results of the present study suggest that the lipid peroxidation-induced ER stress is involved in the anti-breast cancer effects of MAG-DHA. Many studies have reported that n-3 PUFAs induced apoptosis by triggering the intrinsic mitochondrial and ER pathways [22, 23]. In the present study, by using Western Blot and TEM assay, we confirmed that MAG-DHA induced apoptosis in the in vivo tumor xenografts. It has been reported that ER stress has been found to activate caspase-3 apoptotic signaling via caspase-12 [24]. Our results showed that increased expression of caspase-12, cleaved caspase-3 and cleaved PARP was also observed in MAG-DHA-treated breast cancer cells as well as in tumor tissues from MAG-DHA group, and the use of 4-PBA protected the cells from MAG-DHA-induced cell death, further confirming the involvement of ER stress in MAG-DHA-induced apoptotic cell death. It is speculated that MAG-DHA may preferentially increased ROS in or near the plasma membrane lipid rafts, and triggered lipid peroxidation-mediated ER stress, which led to cell apoptosis. At the same time, we could not exclude the involvement of mitochondrial pathway in MAG-DHA-induced apoptosis. In line with our results, Connolly et al. reported that dietary DHA, the sole n-3 PUFA found in golden algae oil, depressed the growth of mammary carcinoma in athymic mice through apoptosis induction [25].

In this study, we found that MAG-DHA not only induced apoptosis but it also induces autophagy as evidenced by increased autophagosome formation in the in vivo tumor xenografts. In addition, we found that autophagy markers LC3- and Beclin1 were significantly up-regulated and p62 was markedly suppressed in MAG-DHA-treated breast cancer cells as well as in tumor tissues from MAG-DHA group. In line with our results, n-3 PUFAs have been found to induce cell autophagy in human pancreatic cancer cells, human multiple myeloma cells and breast cancer cells [22, 26-28]. Autophagy is known as a catabolic process that removes damaged cellular components to maintain cellular integrity and stability under certain conditions, such as in response to nutrient deprivation, organelle damage, or other stresses [29]. However, excessive or prolonged autophagy may also induce cell death [30]. In addition, recent investigations have revealed that ER stress can either stimulate or inhibit autophagy [31, 32]. In this study, it was found that MAG-DHA-induced ER stress triggered autophagy and protected breast cancer cells from MAG-induced apoptotic cell death, suggesting that a combination of MAG-DHA with autophagy inhibitor provides a useful strategy in increasing the therapeutic efficacy in breast cancer.
5. Conclusions

As summarized in Figure 6, our results demonstrated that MAG-DHA exerted its anti-breast cancer effects in cultured breast cancer cells and in in vivo tumor xenografts. The prevention of MAG-DHA against breast cancer was strongly associated with lipid peroxidation-mediated ER stress, which induced cell apoptosis and autophagy. Whereas MAG-DHA induced autophagy activation suppressed apoptotic cell death. Our study also emphasizes the ongoing need for additional preclinical studies investigating the molecular targets of MAG-DHA in breast cancer cells as well as the urgent need for new clinical studies, evaluating the potential role of MAG-DHA supplementation, mainly in combination with autophagy inhibitors, chemo- and radio-therapeutic anticancer regimens, in the improvement of breast cancer patients’ clinical outcome and survival.

Abbreviations

MAG-DHA: Docosahexaenoic acid monoglyceride; MDA: Malondialdehyde; ER: Endoplasmic reticulum; BCA: Bicinchoninic acid; TAG-DHA: DHA-triglycerol; EE-DHA: DHA-ethyl ester; Nrf2: NF-E2-related factor 2; HO-1: Heme Oxygenase 1; PERK: Protein kinase R-like ER kinase; CHOP: C/EBP homologous protein; TEM: Transmission electron microscopy; NAC: DMEM: N-acetyl-L-cysteine; Dulbecco's Modified Eagle's Medium.

Declarations

Ethics approval and consent to participate

All procedures performed in studies involving mice were in accordance with the approved protocol by the Animal Care and Use Committee of the Ningbo University (Ningbo, China)

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

All authors declare no competing interest.

Funding

This work was supported by National Key R&D Program of China (grant no.2018YFD0901105), Zhejiang Province Public Welfare Technology Application Research Project (grant no.LGN19C200010, LGN20C200013), and partly sponsored by the Fang Runhua Fund of Hong Kong, the K.C. Wong Magna
Fund of Ningbo University, Zhejiang Key Laboratory of Pathophysiology (grant no.201802), the Ningbo Civil Natural Science Foundation of China (grant no.2018A610377, 2018A610370 and 2019A610253), the Ningbo Public Welfare Technology Application Research Project (grant no.2019C10032, 2019C10028) and the Medical Science and Technology of Zhejiang province (grant no.2018KY726).

**Authors' contributions**

All authors read and approved the final manuscript. ZQZ and WGY designed overall research experiments. JJZ, YY and FW performed the experiments and data analysis. YY collected and analysed references: JJZ wrote the manuscript. All authors read and approved the manuscript.

**Acknowledgements**

Not applicable.

**References**


Figures
MAG-DHA-induced apoptotic cell death was associated with lipid peroxidation-mediated ER stress. (A) E0771 and SKBR3 cells were treated with MAG-DHA in indicated concentrations alone or in the co-presence of 5mM N-acetyl-L-cysteine (NAC) for 72 h, and cell viability was analyzed using the MTT assay. (B) E0771 and SKBR3 cells were treated with 40 μM MAG-DHA alone or in the co-presence of 5 mM NAC for 48 h, and the content of MDA was determined by MDA kit. (C&D) E0771 and SKBR3 cells were treated with 40 μM MAG-DHA alone or in the co-presence of 5 mM NAC, and the protein expression of oxidative stress response marker Nrf2 and HO-1(C), the ER stress markers such as p-PERK, PERK, p-eIF2α and CHOP was monitored by Western blot analysis. (E) E0771 and SKBR3 cells were treated with MAG-DHA (40μM) alone or in the co-presence of 2.5 mM 4-PBA (an ER stress inhibitor) for 72 h, and cell viability was analyzed using the MTT assay. (F) E0771 and SKBR3 cells were treated with 40 μM MAG-DHA alone or in the co-presence of 2.5 mM 4-PBA, and the protein expression of the apoptotic markers such as caspase-12, cleaved caspase-3 and cleaved PARP was measured. Results are presented as the mean ± SEM, * P < 0.05; ** P < 0.01 by Newman-Keuls test.
MAG-DHA-induced ER stress triggered cell autophagy. (A) E0771 and SKBR3 cells were treated with 40 μM MAG-DHA alone or in the co-presence of 2.5mM 4-PBA, and the protein expression of the autophagic markers such as LC3-II, p62 and Beclin1 were monitored by Western blot analysis. (B) Protein expression levels of Beclin1 after siRNA treatment against Beclin1 in E0771 and SKBR3 cells. (C) siScramble and Beclin1 siRNA-treated E0771 and SKBR3 cells were treated with MAG-DHA (40 μM) for 48 h. and cell viability was analyzed using the MTT assay. (D) E0771 and SKBR3 cells were treated with MAG-DHA (40 μM) alone or in the co-presence of 5 μM chloroquine (CQ) for 48 h, and cell viability was analyzed using the MTT assay.
Figure 3

Effects of MAG-DHA on the growth of E0771 cell xenografts. (A) Representative tumor photographs showing tumor formation at two different time points after cell implantation. (B) E0771 cells (2×10^6 cells/100 μl serum-free RPMI 1640) were subcutaneously injected into C57/BL6 mice as described in the Materials and Methods Section. Tumor size was measured every 3 days, and tumor volume was calculated on the basis of the following formula: tumor volume = (length × width 2) ×0.5. The points
represent mean tumor volume ± SEM obtained from 10 Ctrl mice or 10 MAG-DHA-treated animals. * P < 0.05; ** P < 0.01 compared to the corresponding control.

**Figure 4**

MAG-DHA intervention induced apoptosis and autophagy in the E0771 cell xenografts. (A) The apoptotic markers such as cleaved caspase-3 and cleaved PARP were monitored by Western blot analysis in tumor tissues from Ctrl and MAG-DHA group. (B) The tumor tissues were fixed and analyzed for apoptosis induction using the transmission electron microscopy (TEM). White arrows indicated chromatin marginalization in E0771 cells from MAG-DHA group. (C) The autophagic markers such as LC3-II/I, p62 and Beclin1 were monitored by Western blot analysis in tumor tissues from Ctrl and MAG-DHA group. (D) The tumor tissues were fixed and analyzed for autophagosome formation using the transmission electron microscopy (TEM). White arrows indicated autophagosome formation in E0771 cells from Ctrl and MAG-DHA group. Results are presented as the mean ± SEM, * P < 0.05; ** P < 0.01 compared to the corresponding control.
MAG-DHA intervention induced lipid peroxidation, activated anti-oxidative stress response and triggered ER stress in E0771 xenografts. (A) DHA levels in tumor tissues from Ctrl and MAG-DHA group. (B) The content of MDA was measured by MDA kit in tumor tissues from Ctrl and MAG-DHA group. (C) The sensors of oxidative stress Nrf2 and HO-1 were monitored by Western blot analysis in tumor tissues from Ctrl and MAG-DHA group. (D) The ER stress markers such as p-PERK, PERK, p-eIF2α and CHOP were measured by Western blot analysis in tumor tissues from Ctrl and MAG-DHA group. Results are presented as the mean ± SEM, * P < 0.05; ** P < 0.01 compared to the corresponding control.
Figure 6

Schematic depicting the anti-cancer mechanism of MAG-DHA on breast cancer. The prevention of MAG-DHA against cultured breast cancer cells or in vivo tumor xenografts was strongly associated with lipid peroxidation-mediated ER stress, which induced cell apoptosis and autophagy. Notably, ER-induced autophagy suppressed MAG-DHA-mediated apoptotic cell death.