Introduction

Epithelial ovarian cancer is the leading cause of death among gynecologic malignancies in developed nations. Treatment success of recurrent ovarian cancer is limited by the emergence of chemoresistance. Chemoresistance commonly results from dysregulation of the apoptotic pathway. Therefore, agents that are identified to exert a novel cell death mode may have the potential to be effective against chemoresistant diseases.

Autophagy is primarily considered a process of protein recycling. Recently, interest in autophagy has been renewed in antitumor investigations because various anticancer drugs can induce autophagy in different types of cancer cells. This type of non-apoptotic cell death is considered programmed cell death type II, which is mainly characterized by morphological changes, such as numerous autophagic vacuoles in the cytoplasm. In contrast, apoptosis is classified as programmed cell death type I. Many studies have demonstrated that the two types of cell death are predominantly distinctive but also that cross-talk exists between them. In multiple studies, autophagy has been inhibited, resulting in contrasting outcomes, ie, survival or death, depending on the drugs and cell types analyzed. Interestingly, several tumor suppressors, such as p53, Beclin 1, and PTEN, also play an important role in autophagy. Taken together, these accumulating data may lead to the development of a new cancer therapeutic strategy via drug-induced autophagy.

Fucoxanthin (Figure 1A) has a unique structure, including an allenic bond and a 5,6-monoepoxide, and is a major marine carotenoid found in edible seaweeds, such as Undaria pinnatifida, Hijikia fusiformis, and Sargassum fulvellum. Recent studies have demonstrated that fucoxanthin has antitumor effects, promotes apoptotic effects, anti-inflammatory effects, and radical scavenging activity.

Although it is often controversial whether autophagy in cancer cells causes cell death or cell protection, our results...
demonstrate that treatment with 3-methyladenine, which is a phosphatidylinositol 3-phosphate kinase inhibitor, not only reverses fucoxanthin-mediated autophagy but also partially reverses the cytotoxic effect of fucoxanthin, thereby suggesting that autophagy is the vital factor in fucoxanthin-mediated cytotoxic effects in HeLa cells. Mechanistically, fucoxanthin-mediated autophagy is dependent on the Akt/mTOR signaling pathway.

Materials and methods

Materials
RNase A, 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT), acridine orange (AO), fucoxanthin, bafilomycin A1 (Baf A1), ammonium chloride (NH4Cl), 3-methyl adenine (3-MA), propidium iodide (PI) and a monoclonal anti-p62 antibody were purchased from Sigma (St Louis, MO, USA). RPMI-1640 and fetal calf serum (FCS) were obtained from Gibco (Grand Island, NY, USA). An AnnexinV-FITC apoptotic detection kit and primary antibodies against phospho-Akt, Akt, p21, PTEN, p53, CDK2, cyclin D1, phospho-p70S6 kinase (p-p70S6K; Thr389) and phospho-mTOR (p-mTOR) were purchased from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies against Beclin 1, LC3, and cathepsin D as well as horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). LysoTracker Red and Hoechst 33342 were purchased from Biyuntian (Haimen, China). All other chemicals used in the experiments were commercial products of reagent grade.

Cell culture and cytotoxicity assay
HeLa cells were purchased from Shanghai Institute for Biological Science (Shanghai, China) and were supplemented with 1 mmol/L glutamine and 10% FCS. The cells were cultured at 37 °C under 5% CO2 atmosphere.

The cytotoxic effect of fucoxanthin was evaluated in HeLa cells by the conversion of MTT to a purple formazan precipitate, as previously described[11]. After cells were seeded into 96-well plates at 5000 cells/well for 24 h, fucoxanthin was added, and the cells were incubated for an additional 48 h. The inhibition rate was calculated from plotted results using untreated cells as 100%[11].

Cell cycle and cellular apoptotic evaluation
After treatment with fucoxanthin for 48 or 72 h, the cells were fixed with ice-cold 70% ethanol and then stained with PI (100 μg/mL) after removing the RNA from the cells by RNase A treatment (50 μg/mL). The analysis was performed using a FACSscan (Becton Dickinson, USA) emitting at 488 nm. Data acquisition and analysis were controlled by Modifit software[12].

Cell apoptosis was evaluated by an AnnexinV-FITC apoptotic detection kit using FACSscan (Becton Dickinson, USA). Briefly, the cells were seeded in six-well plates, exposed to fucoxanthin for 48 h, harvested and stained according to the manufacturer’s protocol[12].

Autophagy detection with acridine orange staining
As a marker of autophagy, the volume of the cellular acidic compartment was visualized by acridine orange staining[33]. Cells were seeded into 6-well plates and treated as described above for the cytotoxicity assay. After 48 h of treatment, cells were incubated with medium containing acridine orange (1 mg/mL) for 15 min. For autophagy inhibitor analysis, cells were pretreated with 3-MA (5 mmol/L) for 2 h. Acridine orange was removed, and fluorescent micrographs were taken using a fluorescent microscope. A shift from green to red fluorescence indicated acidic vesicles consistent with autolysosomes. Western blotting analysis was performed for LC3-II and Beclin 1, which is an essential autophagy-related protein[12].

Lysosome detection with LysoTracker Red staining
After the fucoxanthin incubation, cells were stained with LysoTracker Red (50 nmol/L), a specific red fluorescent dye for lysosomes, for 45 min at 37 °C, and the cells were then counterstained with Hoechst 33342 (1 μmol/L) for 15 min in the dark. Fluorescent micrographs were taken using a fluorescence microscope[12].

Transient transfection and autophagy assays
HeLa cells were plated on coverslips at a density of 2×10⁵ cells/coverslip and cultured to 60% confluence. Transient transfection of 2 μg/mL GFP-LC3 plasmid DNA in each dish was performed using Lipofectamine 2000 according to the manufacturer’s protocol. After incubation in Opti-MEM medium for 6 h, the cells were incubated in RPMI-1640 containing 10% FBS. Autophagy was examined by analyzing the formation of fluorescent puncta of autophagosomes in GFP-LC3-transfected cells using fluorescence microscopy. When cells reached 90% confluence, fucoxanthin was added to the culture medium. After a 48 h treatment, the cells were fixed with 4% paraformaldehyde. The GFP-LC3 punctate dots per cell in GFP-LC3-positive cells were counted. Thirty cells per slide were counted, and three slides were used for each condition[14].

Western blotting
After fucoxanthin treatment, HeLa cells were harvested and washed with PBS. Total cellular protein was isolated using a protein extraction buffer (containing 150 mmol/L NaCl; 10 mmol/L Tris, pH 7.2; 5 mmol/L EDTA; 0.1% Triton X-100; 5% glycerol; and 2% SDS). Protein concentration was determined using a protein assay kit (Beyotime, Haimen, China). Equal amounts of protein (50 μg/lane) were separated using 8% SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with primary antibodies. After washing with PBS, the membranes were incubated with corresponding peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies followed by detection with enhanced chemiluminescence staining. β-Actin (45 kDa; cytosolic protein) was used to normalize protein loading[12]. The level of protein expression was quantified by densitometry analysis.
using a gel-imaging system.

Statistical analysis
All data are presented as the mean±SD and were analyzed using analysis of variance (ANOVA) followed by a q-test.

Results
Fucoxanthin exerted potent cytotoxic activity but did not induce cell apoptosis
After treatment for 48 h, the cytotoxic activity of fucoxanthin was significantly increased in a dose-dependent manner in HeLa cells. The IC₅₀ value of fucoxanthin was 55.1±7.6 μmol/L (Figure 1B). Flow cytometric analysis demonstrated that fucoxanthin induced cell cycle arrest at the G₀/G₁ phase in a dose-dependent manner (Figure 1C). Further investigation showed that fucoxanthin increased the expression of p21 and decreased the expression of cyclin D1 and CDK2, which are G₁-related proteins (Figure 4). Next, we investigated if fucoxanthin-induced cell death was caused by apoptosis. Annexin V/PI double labeling was used for the detection of PS externalization, a hallmark of early phase apoptosis. Compared to control cells, the percentage of annexin V-positive cells was not significantly increased after a 48 or 72 h treatment with fucoxanthin (Figure 1D), thereby suggesting that fucoxanthin did not induce HeLa cell apoptosis.

Fucoxanthin induced HeLa cell autophagy
Autophagy can cause cell growth inhibition. The association of autophagic vacuole formation with autophagic cell death was evaluated. Based on acidine orange staining, there was a significant increase in autophagic vesicle (red fluorescence) formation in HeLa cells after being exposed to fucoxanthin, as shown in Figure 2A. During the process of autophagic vesicle formation, the number and activity of lysosomes increase; therefore, we further examined lysosomes using LysoTracker Red, a specific red fluorescent dye for lysosomes. As shown in Figure 2B, the fluorescence of LysoTracker Red increased after treatment with fucoxanthin in a dose-dependent manner. Furthermore, Western blotting results and quantitative analysis demonstrated that treatment with fucoxanthin for 48 h markedly upregulated Beclin 1 (initiation factor for autophagosome formation) and promoted the conversion of LC3 I to LC3 II (autophagosome marker) as compared to the control (Figure 2C). These results suggested that fucoxanthin induced autophagy in HeLa cells after treatment for 48 h.

Furthermore, the occurrence of autophagy after fucoxanthin exposure in HeLa cells was also validated by detecting the expression of GFP-LC3. Following fucoxanthin treatment, GFP-LC3-transfected cells showed punctate green fluorescence at 48 h. In contrast, control transfected cells without fucoxanthin treatment showed a diffuse distribution of green fluorescence (Figure 2D). These findings further demonstrated that fucoxanthin induced HeLa cell autophagy.

To assess autophagic flux, Baf A1, which is a potent and specific inhibitor of vacuolar H⁺-ATPases, was used to suppress the acidification of the lysosome and the autophagosome/lysosome[15]. As shown in Figure 3A, Baf A1 significantly enhanced fucoxanthin-mediated GFP-LC3 patches. Western blot analysis also demonstrated that the fucoxanthin-induced upregulation of LC3 II dramatically increased in the presence...
Figure 2. Fucoxanthin induced HeLa cell autophagy after treatment for 48 h. (A) Representative micrographs of acridine orange staining of HeLa cells after treatment with fucoxanthin (Magnification ×20). (B) Representative micrographs of LysoTracker Red and Hoechst 33342 staining of HeLa cells after treatment with fucoxanthin (Magnification ×20). (C) Protein expression of Beclin-1 and LC3 in HeLa cells was detected by Western blotting after treatment with fucoxanthin for 48 h. (D) Representative Hoechst 33342 staining micrographs of transiently transfected HeLa cells with GFP-LC3 after treatment with fucoxanthin (Magnification ×20). Arrowheads indicate GFP-LC3 puncta.

Figure 3. Autophagic flux was detected after treatment with fucoxanthin for 48 h. (A) Quantification of autophagy in fucoxanthin-treated HeLa cells transfected with GFP-LC3 in the presence or absence of 100 nmol/L Baf A1 for 48 h. Western blotting analysis (B) and quantitative analysis (C) of LC3 expression in HeLa cells after treatment with fucoxanthin in the presence or absence of 100 nmol/L Baf A1 for 48 h. Western blotting analysis (D) and quantitative analysis (E) of p62 expression in HeLa cells after treatment with fucoxanthin in the presence or absence of 50 mmol/L NH₄Cl for 48 h. Western blotting analysis (F) and quantitative analysis (G) of cathepsin D expression in HeLa cells after treatment with fucoxanthin for 48 h. Mean±SD. bP<0.05, cP<0.01 compared to the control (n=3).
of Baf A1 (Figure 3B and 3C). To further detect autophagic flux, we treated cells with NH$_4$Cl, an inhibitor of lysosomal acidification, which inhibits autophagic degradation of autophagic substrates. After treatment with fucoxanthin, the expression level of p62 was significantly decreased. However, the addition of NH$_4$Cl caused the levels of p62 to recover, suggesting that p62 was degraded by the autophagy process (Figure 3D and 3E). Autophagy is a constitutive process that includes activation of lysosomal enzymes and subsequent degradation of their substrates. Therefore, the expression levels of cathepsin D, which is an aspartic protease localized inside the lysosomes, is increased during autophagy. Our results demonstrated that cathepsin D was upregulated after fucoxanthin treatment.

**Fucoxanthin reduced phosphorylated Akt and its downstream targets**

Recent studies have shown that the inhibition of Akt and its downstream target, mTOR, contribute to the initiation of autophagy[16]. Once activated, Akt transduces signals to downstream targets that control cell survival and autophagy[17]. To assess the involvement of the Akt pathway in fucoxanthin-induced autophagy, the related proteins were investigated by Western blotting. As shown in Figure 4, phosphorylated Akt significantly decreased after a 48 h treatment with fucoxanthin in a dose-dependent manner. In addition, we investigated the expression of downstream target proteins of Akt. Fucoxanthin significantly inhibited the phosphorylation of p53, p70S6K and mTOR, and it upregulated the expression of PTEN, thereby suggesting that the Akt signaling pathway was involved in fucoxanthin-mediated autophagy.

**3-MA reversed cytotoxic effects and fucoxanthin-mediated autophagy**

To investigate if the cytotoxic activity of fucoxanthin was dependent on autophagy, HeLa cells were pretreated with 3-MA before administration of fucoxanthin. As shown in Figure 5A, 3-MA partially reversed the fucoxanthin-mediated cytotoxic effects. In addition, fucoxanthin did not induce apoptosis in HeLa cells pretreated with 3-MA (Figure 5B). 3-MA also reversed fucoxanthin-mediated autophagy and inhibited the Akt signaling pathway (Figure 5C-5G), thereby suggesting that the antitumor activity of fucoxanthin was dependent on fucoxanthin-induced autophagy.

**Discussion**

The role of the autophagic process in antitumor therapy has not been clearly elucidated[18, 19]. After treatment with antitumor drugs, some cancer cells undergo autophagy as a temporary survival mechanism, and the suppression of autophagy leads to apoptosis, thus enhancing antitumor effects. In contrast, several antitumor drug treatments, including some chemotherapeutic agents, have been reported to induce autophagic cell death[3, 20]. In the present study, fucoxanthin induced HeLa cell autophagy but not apoptosis, even with 3-MA pretreatment.
and that 3-MA reversed these effects. However, fucoxanthin did not induce HeLa cell apoptosis using the same dose and treatment time, even with 3-MA pre-incubation, thereby suggesting that fucoxanthin only induced HeLa cell autophagy.
and not apoptosis. Efficient autophagy is dependent on the balance between the formation and elimination of autophagosomes, and a deficit in any part of this process will cause autophagic dysfunc-
tion. Thus, autophagy flux should be detected in fucoxanthin-
mediated HeLa cell autophagy. Our results demonstrated that fucoxanthin enhanced autophagy flux, including an increase in autophagosome formation, lysosome activation and de-
gradation of autophagic substrates. This effect was different from the effect of perifosine, which inhibits autophagy and enhances apoptosis[29]. However, Tafuku et al demonstrated that fucoxanthin induces B-cell malignancies, such as Hodg-
kin’s lymphoma, Burkitt’s lymphoma and Epstein-Barr virus-
immortalized B-cells, through apoptosis by suppressing the NF-κB signaling pathway and cell cycle arrest at the G₁ phase; however, they reported that fucoxanthin did not induce apop-
tosis in normal peripheral blood mononuclear cells[20].

3-MA partially reversed the fucoxanthin-mediated cytotoxic effects, suggesting that the antitumor activity of fucoxanthin was autophagy-dependent. Several reports indicated that Akt/mTOR signaling negatively regulates autophagy through mTOR, which is a downstream target of Akt[26, 27]. In the present study, we demonstrated that fucoxanthin inhibited Akt/mTOR signaling, as demonstrated by the inhibition of the phosphorylation of Akt, p70S6K and mTOR, resulting in the conversion of LC3 I to LC3 II, a hallmark of autophagy. In the context of induction of autophagy, Aoki et al showed that natural products induce autophagy through the inhibition of Akt/mTOR signaling, which agreed with the present results[16].

Autophagy is a signal transduction pathway that can affect the G₁ phase progression. The autophagic process during G₁ arrest can repair cell damage to avoid cell death[29]. Some inhibitors of the Akt signaling pathway, such as NVP-
BEZ235, induce tumor cell autophagy and cell cycle arrest[20]. Our results demonstrated that fucoxanthin induced cell cycle arrest at the G₀/G₁ phase via inhibiting the AKT signaling pathway and that fucoxanthin also regulated the expression of cell cycle-related proteins by upregulating p21 expression and downregulating CDK2 and cyclin D1 expression. These results were similar to previous reports that fucoxanthin induces cell cycle arrest at the G₁ phase but not apoptosis in LNCap[30], HepG2 and DU145 cells[31]. However, Satomi reported that fucoxanthin induces LNCap prostate cancer cell cycle arrest at the G₁ phase via SAPK/JNK signal pathway activation[30]. In addition, Yoshihiko and Hoyokou found that fucoxanthin induces HepG2 and DU145 cell cycle arrest at the G₁ phase via induced GADD45A, a cell cycle-related gene. Accordingly, these data suggest that fucoxanthin-mediated tumor cell death and its molecular mechanism depend on the tumor cell type.

Recently, as a potential therapeutic approach for malignant tumors, targeting of the Akt/mTOR pathway has been sug-
gested in the field of chemotherapy[28]. Hence, our results also support consideration of the potential use of fucoxanthin as an antitumor agent because fucoxanthin mediates autophagy via inhibition of the Akt/mTOR signaling pathway in HeLa cells.

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Author contribution
Prof Song-qiang XIE designed the research and revised the manuscript; Li-li HOU and Chao GAO conducted the research; Liang CHEN helped with portions of the research; and Guo-
qiang HU performed the statistical analysis.

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