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ARTICLE

Osthole Suppresses Fatty Acid Synthase Expression in HER2-Overexpressing Breast Cancer Cells through Modulating Akt/mTOR Pathway

Victor Chia-Hsiang Lin,[†] Chun-Hung Chou,[‡] Ying-Chao Lin,[§,∥] Jia-Ni Lin,[⊥] Chien-Chih Yu,[#] Chih-Hsin Tang,[▽] Hui-Yi Lin,*[,#] and Tzong-Der Way*[,‡]

[†]Department of Urology, E-Da Hospital, Department of Nursing, I-Shou University, Kaohsiung, Taiwan, [‡]Department of Biological Science and Technology, College of Life Sciences, China Medical University, Taichung, Taiwan, [§]Institute of Biochemistry, College of Life Science, National Chung Hsing University, Taichung, Taiwan, [∥]Division of Neurosurgery, Buddhist Tzu Chi General Hospital, Taichung Branch, [⊥]The Ph.D. Program for Cancer Biology and Drug Discovery, College of Pharmacy, China Medical University, Taichung, Taiwan, [#]School of Pharmacy, College of Pharmacy, China Medical University, Taichung, Taiwan, and [▽]Department of Pharmacology, College of Medicine, China Medical University, Taichung, Taiwan

While fatty acid synthase (FASN) has been shown to be expressed in many human solid tumors, FASN has also been identified in preneoplastic lesions. HER2, which has also been identified in preneoplastic breast lesions, has been shown to upregulate FASN expression. Osthole, an active constituent isolated from the fruit of *Cnidium monnieri* (L.) Cusson, a traditional Chinese medicine, was found to be effective in suppressing FASN expression in HER2-overexpressing breast cells. Osthole preferentially inhibited proliferation and induced apoptosis in HER2-overexpressing cancer cells. Moreover, osthole inhibited the phosphorylation of Akt and mTOR. The use of Akt-overexpression revealed that the modulation of Akt and mTOR was required for osthole-induced FASN suppression. Finally, we showed that osthole could enhance paclitaxel-induced cytotoxicity in HER2-overexpressing cancer cells. These results suggested that osthole has the potential to advance as chemopreventive or chemotherapeutic agent for cancers that overexpress HER2.

KEYWORDS: Fatty acid synthase; osthole; apoptosis; cytotoxicity

INTRODUCTION

The high level of fat in the Western diet has been related to the development of many human solid tumors including colon, breast, and ovarian carcinoma (*1*). Fatty acid synthase (FASN, EC 2.3.1.85) is a multifunctional enzyme that performs seven sequential reactions to convert acetyl-CoA and malonyl-CoA to palmitate and plays a central role in the anabolic conversion of dietary calories into a storage form of energy in mammals (*2*). Numerous clinical and basic studies indicate that increased expression of FASN has emerged as a common phenotype in breast cancer and in varieties of human solid tumors, particulary those with a poor prognosis (*3*). Recent studies have demonstrated that inhibition of FASN with pharmacological inhibitors is selectively cytotoxic to human cancer cell and leads to a significant antitumor effect (*4*), suggesting that activation of fatty acid synthesis is required for carcinogenesis. Collectively, overexpression of FASN occurs in a wide variety of human cancers and is associated with poor prognosis, suggesting that fatty acid synthesis provides an advantage for tumor growth, and could be a promising target for chemoprevention or chemotherapy.

Breast cancer and ovarian cancer remain the most common malignancies diagnosed in women in Western society (*5*). In up to 30% of human breast cancers and ovarian cancer was found overexpression of the HER2 (alternatively known as *neu* or erbB2) receptor or amplification of the HER2 gene, or both (*6*). HER2 is a member of the class I (epidermal growth factor receptor) family of transmembrane receptor tyrosine kinases. Overexpression of the HER2 receptor has been shown to be correlated with poor prognosis, enhanced metastatic potential, and increased chemoresistance of human cancers (*7*). HER2 has been shown to regulate FASN transcriptional expression through phosphatidylinositol 3-kinase (PI3K)/Akt signal transduction pathways, enlightening a molecular connection between FASN and HER2 in human breast cancer cells (*8*). Especially, inhibition of FASN preferentially induced apoptosis of HER2-overexpressing cancer cells (*9*) and delayed tumor progression in *neu*-N transgenic mice (*10*), suggesting that upregulation of FASN expression might play a role in HER2-mediated cell survival and tumorigenesis. Taken together, targeting FASN might be a novel approach to treat or prevent HER2-overexpressing cancer.

Recently, there has been a global trend toward the use of natural matters present in fruits, vegetables, and herbs as antioxidants and

*Corresponding authors. T.-D.W.: tel, (886)-4-2205-3366, ext 2509; fax, (886)-4-2207-0465; e-mail, tdway@mail.cmu.edu.tw. H.-Y.L: tel, +886-4-2205-3366, ext 5112; fax, +886-4-2203-1075; e-mail, hylin@mail.cmu.edu.tw.

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anticancer agents (*11*). The fruit of *Cnidium monnieri* (L.) Cusson, a well-known traditional Chinese medicine, possesses a variety of pharmacological properties and has been administered to humans in clinics for many years. Osthole [7-methoxy-8-(3-methyl-2-butenyl)-2*H*-1-benzopyran-2-one] is an active compound isolated from the fruit of *Cnidium monnieri* (L.) Cusson. Osthole has received considerable attention recently because of its significant and diverse pharmacological and biological uses, which make it a very promising natural lead compound for new drug discovery. Modern pharmacological studies have shown that osthole has many biological functions, including antiosteoporotic (*12*), antihepatitic (*13*), antiallergic (*14*), antiseizure (*15*), and antiproliferative functions (*16*). Moreover, recent studies found that osthole could improve the accumulation of lipids and decrease the lipid levels in serum and hepatic tissue in alcohol-induced fatty liver mice and rats (*17*), but its mechanism was not completely understood.

Based on the facts that FASN expression plays an important role in the survival and tumorigenesis in HER2-overexpressing cancer and that osthole has favorable effects on both lipid metabolism and anticancer, it promotes us to investigate whether osthole could diminish FASN expression in HER2-overexpressing cancer. Examining the various natural components revealed that osthole was most effective suppression of FASN expression in HER2-overexpressing cancer cells. Osthole preferentially inhibited proliferation and induced apoptosis in HER2-overexpressing cancer cells. Furthermore, we showed that the modulation of Akt and mTOR was potentially required for osthole-induced FASN suppression. Finally, osthole could specifically sensitize paclitaxel-induced cytotoxicity in HER2-overexpressing cancer cells. These results indicate that osthole could downregulate FASN expression and induce apoptosis in HER2-overexpressing cancer cells through modulating Akt and mTOR, and further suggest that osthole has therapeutic implications in HER2-overexpressing cancer cells.

MATERIALS AND METHODS

Chemicals. Osthole, paeonol, aristolochic acid, bergapten, fatty acid-free bovine serum albumin (BSA), G418, C75, palmitic acid, and MTT were purchased from Sigma Chemical Co. (St. Louis, MO). Antibody for FASN was purchased from BD Biosciences (Los Angeles, CA). Antibodies for Akt, mTOR, p44/42 MAPK, phospho-mTOR (Ser2448), phospho-Akt (Ser-473), and phospho-p44/p42 MAPK (Thr202/Tyr204) were purchased from Cell Signaling Technology (Beverly, MA). β-Actin antibody was from Abcam Inc. (Cambridge, MA). Anti-mouse and anti-rabbit antibodies conjugated to horseradish peroxidase were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other reagents and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and were of an analytical grade.

Cell Culture. HER2-overexpressing human cancer cell lines, SKOV3 (ovarian carcinoma) and cell lines with low HER2 expression, including MDA-MB-231 (breast adenocarcinoma), MCF-7 (breast adenocarcinoma), and HBL-100, which is derived from a normal human breast tissue transformed by SV40 large T antigen, were obtained from American Type Culture Collection (Manassas, VA). All of the cells were grown in DMEM/F12 supplemented with fetal calf serum (Hyclone Laboratories, Logan, UT) and 1% penicillin−streptomycin. The MCF-7/neo and MCF-7/HER18 transfectants, which were kindly provided by Dr. Mien-Chie Hung (The University of Texas M. D. Anderson Cancer Center, Houston, TX), were cultured as described before (*18*). These cells were grown at 37 °C in a humidified atmosphere of 5% CO_2.

For culturing cells in the presence of palmitate−bovine serum albumin complex, palmitate (Sigma) was first complexed to fatty acid-free bovine serum albumin. Briefly, 4 volumes of a 4% bovine serum albumin solution in 0.9% NaCl were added to 1 volume of 5 mM palmitate in ethanol and incubated at 37 °C for 1 h to obtain a 1 mM stock solution of bovine serum albumin-complexed palmitate.

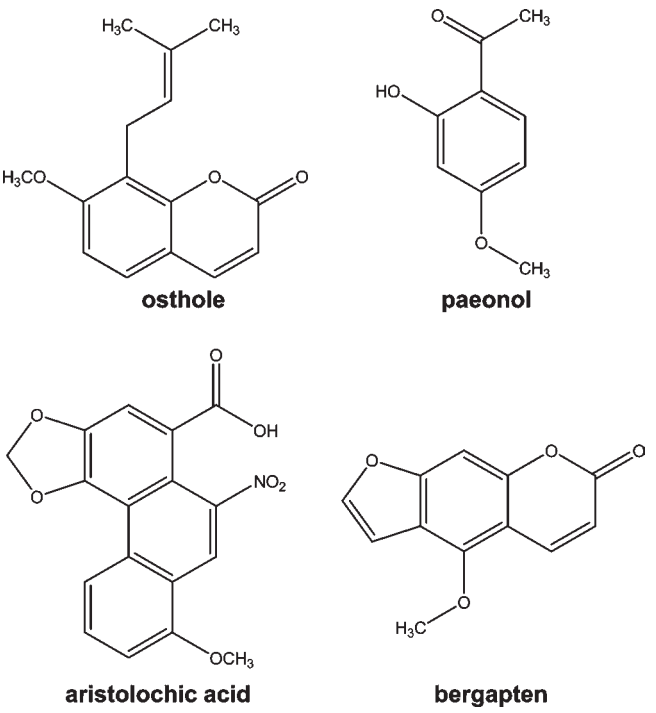

Figure 1. Chemical structures of osthole, paeonol, aristolochic acid and bergapten.

Western Blot Analysis. Cells (1.5×10^6) were seeded onto a 100 mm tissue culture dish containing 10% FBS DMEM/F12 and cultured for 24 h. Then cells were incubated in 10% FBS DMEM/F12 and treated with various agents as indicated in the figure legends. After treatment, cells were placed on ice, washed with cold PBS, and lysed in lysis buffer. Western blot was done as described previously (*19*). The intensity of the bands was scanned and quantified with NIH image software.

Cell Proliferation Assays. As described previously (*20*), the effects of osthole and paclitaxel on cell proliferation were examined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method.

Cell Cycle Analysis. Cells (5×10^5) were cultured in 6 mm cell culture dish and incubated for 24 h. Cells were then harvested in a 15 mL tube, washed with PBS, resuspended in PBS, and fixed in 2 mL of iced 100% ethanol at −20 °C overnight. Cell pellets were collected by centrifugation, resuspended in 0.5 mL of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/mL RNase), and incubated at RT for 30 min. Subsequently, 1 mL of propidium iodide solution (50 μg/mL) was added, and the mixture was allowed to stand on ice for 30 min. Fluorescence emitted from the propidium iodide−DNA complex was quantitated after excitation of the fluorescent dye by FAC-Scan cytometry (BD Biosciences, San Jose, CA).

Tranfection. One day before transfection, 2×10^5 cells without serum and antibiotics were plated in six-well plates. SKOV3 cells were grown to 90% confluence and transfected on the following day by Lipofectamine 2000 (Invitrogene, Carlsbad, CA), premixed plasmid DNA with OPTI-MEM (GIBCO, Carlsbad, CA) for 5 min and then added to each well. After 24 h of incubation, the transfection was completed.

Statistical Analysis. All values were expressed as mean ± SE. Each value is the mean of at least three separate experiments in each group. Student's *t*-test was used for statistical comparison. * indicates the values are significantly different from the control (*, $P < 0.05$; **, $P < 0.01$).

RESULTS

Effects of Osthole, Paeonol, Aristolochic Acid, and Bergapten on the Expression of FASN in HER2-Overexpressing Cancer Cells. Osthole, paeonol, aristolochic acid, and bergapten (**Figure 1**) are naturally occurring plant bioactive compounds and have been shown to have antineoplastic activities. To investigate their inhibitory activities on the protein level of FASN in HER2-overexpressing cancer cell lines, we treated separately the

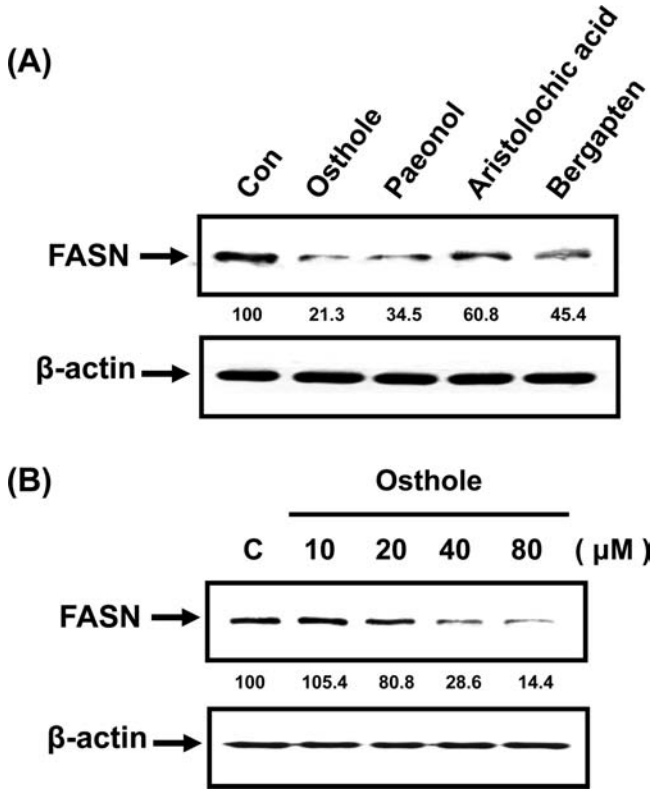

Figure 2. Effects of osthole, paeonol, aristolochic acid and bergapten on the FASN expression in HER2-overexpressing cancer cells. (**A**) SKOV3 cells were incubated with DMSO (Con) or osthole, paeonol, aristolochic acid, bergapten (40 μM) at 37 °C for 48 h. Immunoblotting was used to measure protein levels of FASN and β-actin. (**B**) SKOV3 cells were treated with various concentrations of osthole for 48 h. Immunoblotting was used to measure protein levels of FASN and β-actin. Western blot data presented are representative of those obtained in at least three separate experiments. The values below the figures represent change in protein expression normalized to β-actin.

HER2-overexpressing human ovarian cancer SKOV3 cells with 40 μM of these compounds at 37 °C for 48 h, and the level of FASN protein was analyzed. As shown in **Figure 2A**, among the tested compounds, osthole was the most effective compound in suppressing FASN expression. Next, we assessed the effects of osthole on FASN protein expression. Treatment of SKOV3 cells with osthole resulted in a dose-dependent decrease in protein expression of FASN (**Figure 2B**).

Osthole Preferentially Inhibits the Proliferation of HER2-Overexpressing Cancer Cells. To assess the biological activity of osthole in terms of cell proliferation, MDA-MB-231, MCF-7, and SKOV3 cells were treated with osthole at different concentrations for 48 h. The growth inhibition of the tested cell lines occurred in a dose-dependent manner but to various extents (**Figure 3A**). SKOV3 cells that overexpress HER2 showed higher susceptibility to osthole than did MDA-MB-231 or MCF-7 cells, resulting in lower cell viability.

Osthole Preferentially Promotes Apoptotic Cell Death in HER2-Overexpressing Breast Cancer Cells. We next examined whether the loss of cell viability was due to an increase in apoptotic cell death. The percentage of apoptotic cells in the sub-G1 peak of osthole-treated cells was measured by flow cytometry. As shown in **Figure 3B**, compared with vehicle-treated control, osthole dramatically increased the amount of apoptotic cells (sub-G1 cells) in the HER2-overexpressing cancer cell lines. In contrast, in HER2 low-expressing cell lines, the level of apoptotic cells was similar after either vehicle or osthole treatment. To avoid the genetic complexities associated with established cancer cell lines, we compared the sensitivity of MCF-7/neo cells, which were established by transfecting the neomycin resistance gene alone into MCF-7 cells, with that of isogenic HER2-transfected MCF-7 (MCF-7/HER18). Consistent with our expectations, we found that osthole preferentially induced apoptosis in MCF-7/HER18 cells when compared with MCF-7/neo cells ($p < 0.05$) (**Figure 3C**). Therefore, osthole can preferentially induce apoptosis in HER2-overexpressing cancer cell lines.

Palmitate Suppresses Osthole-Induced Cytotoxicity in HER2-Overexpressing Cancer Cells. To confirm that the cancer cell cytotoxicity induced by osthole was related to FASN inhibition, SKOV3 cells were exposed for 48 h to C75, a synthetic FASN inhibitor, or osthole in the presence of exogenous palmitate (80 μM), the end product of FASN reaction. Palmitate reduced the cytotoxic effects of both FASN inhibitors (**Figure 4**), especially the osthole effects, as the sub-G1 was significantly decreased after the addition of exogenous palmitate ($p < 0.01$).

Osthole Downregulates Phospho-Akt and Phospho-mTOR Levels in HER2-Overexpressing Cancer Cells. Akt and ERK have been shown to be involved in FASN regulation in various cell types (*21*). To examine whether the activities of Akt and ERK are affected by osthole, we analyzed the phosphorylation of Akt and ERK1/2 in SKOV3 cells after treatment with osthole (10−80 μM) for 48 h. Immunoblot analysis with anti-phospho-specific antibody was then performed. As shown in **Figure 5A**,**B**, osthole inhibited the phosphorylation of Akt and its downstream mTOR. In contrast, osthole did not affect ERK1/2 phosphorylation. If Akt was a critical target of osthole, profound expression of Akt should attenuate osthole inhibited FASN expression. To assess this hypothesis, we performed an Akt-overexpression experiment using cDNA of CA-Akt. As a result, Akt-overexpression rendered osthole unable to suppress FASN expression (**Figure 5C**) and also inhibited osthole's ability to induce apoptosis (**Figure 5D**). Taken together, it suggests that osthole inhibits FASN expression by repressing the phosphorylation of Akt and mTOR in SKOV3 cells.

Osthole Can Enhance the Chemosensitivity of HER2-Overexpressing Cancer Cells. Previous studies have shown that HER2 overexpression can confer resistance to chemotherapeutic agents in cancer cells (*22*) and that inhibition of FASN can sensitize HER2-overexpressing cancer cells to paclitaxel (*23*). Because osthole can inhibit FASN expression in HER2-overexpressing cancer cells, we thus evaluated whether osthole could sensitize HER2-overexpressing cancer cells to the chemotherapeutic agent paclitaxel. To investigate whether osthole may sensitize HER2-overexpressing cancer cells to the clinical drug paclitaxel, we examined the treatment of osthole alone and osthole combined with paclitaxel on growth of cancer cells. As the MTT assay data shown, the HER2-overexpressing cancer cells SKOV3 (**Figure 6A**) were much more resistant to paclitaxel than HER2 low expressed level breast cancer cells MDA-MB-231 (**Figure 6B**) and MCF-7 (**Figure 6C**). In the efficacy of combinational condition, the cytotoxicity of osthole combined with paclitaxel for HER2-overexpressing cancer cells was obviously more increased than in the case of treatment by osthole or paclitaxel alone. The results indicate that osthole enhances the cytotoxicity effect of paclitaxel on HER2-overexpressing cancer cells and reduces the HER2 induced drug resistance of cancer cells. Therefore, the function of osthole may improve the efficacy and sensitivity of paclitaxel for therapy in HER2-overexpressing cancer cells.

DISCUSSION

In Asia, complementary and alternative medicine (CAM) therapies such as traditional Chinese medicine have been an

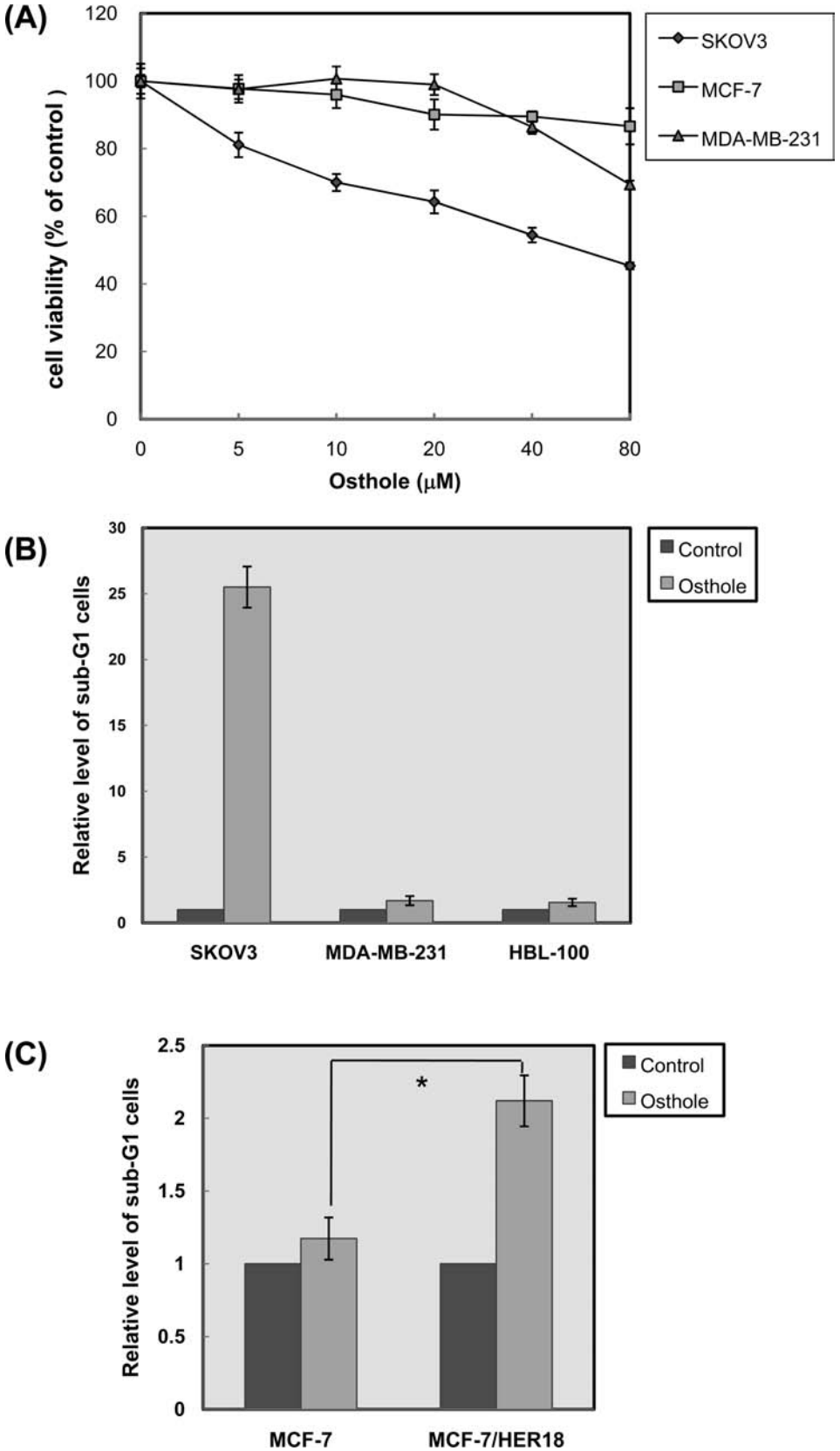

Figure 3. Osthole induces apoptosis in HER2-overexpressing cancer cell lines but not in HER2 low-expressing cancer cell lines. (**A**) SKOV3, MCF-7 and MDA-MB-231 cells were treated with various concentrations of osthole at 37 °C for 48 h, and cell viability was determined by MTT assays. The number of viable cells after treatment is expressed as a percentage of the vehicle-only control. Data are means of three independent experiments. Bars represent the SE. (**B**) SKOV3, MDA-MB-231 and HBL-100 cells were treated with vehicle or osthole (40 μM) for 48 h, then harvested and subjected to sub-G1 DNA content analysis. (**C**) MCF-7/neo (MCF-7) or MCF-7/HER18 cells were treated with vehicle or osthole (40 μM) for 48 h, then harvested and subjected to sub-G1 DNA content analysis. The level of apoptosis induced by vehicle was set at 1, and the level of apoptosis induced by osthole was expressed relative to this value. Columns, average of three independent experiments; bars represent the SE. *, $p < 0.05$.

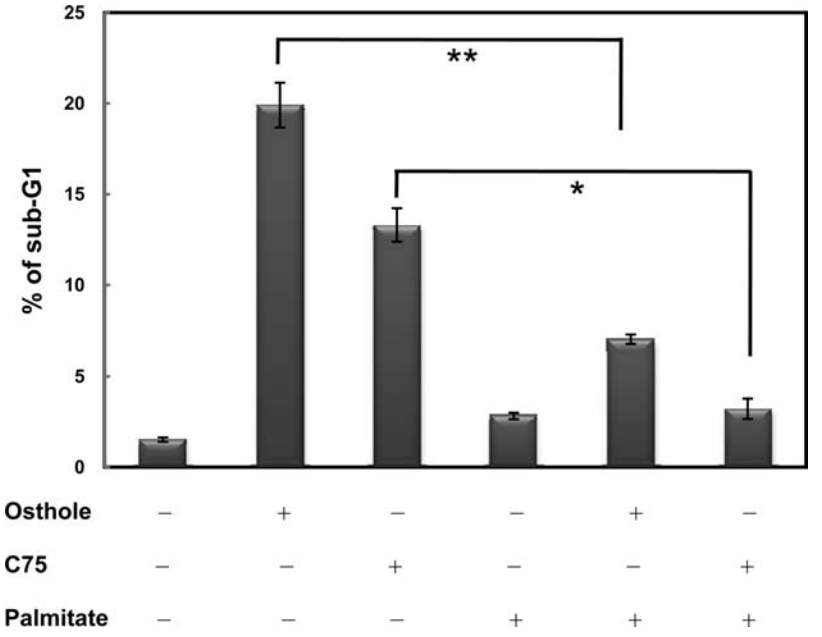

Figure 4. Exogenous palmitate suppresses osthole-induced cancer cell cytotoxicity. SKOV3 cells were exposed to vehicle (control) or osthole (40 μM), C75 (30 μM) in the presence or absence of 80 μM palmitate. After 48 h, cells harvested and subjected to sub-G1 DNA content analysis. The indicated percentages are the mean of three independent experiments. Bars represent the SE. *, $p < 0.05$; **, $p < 0.01$.

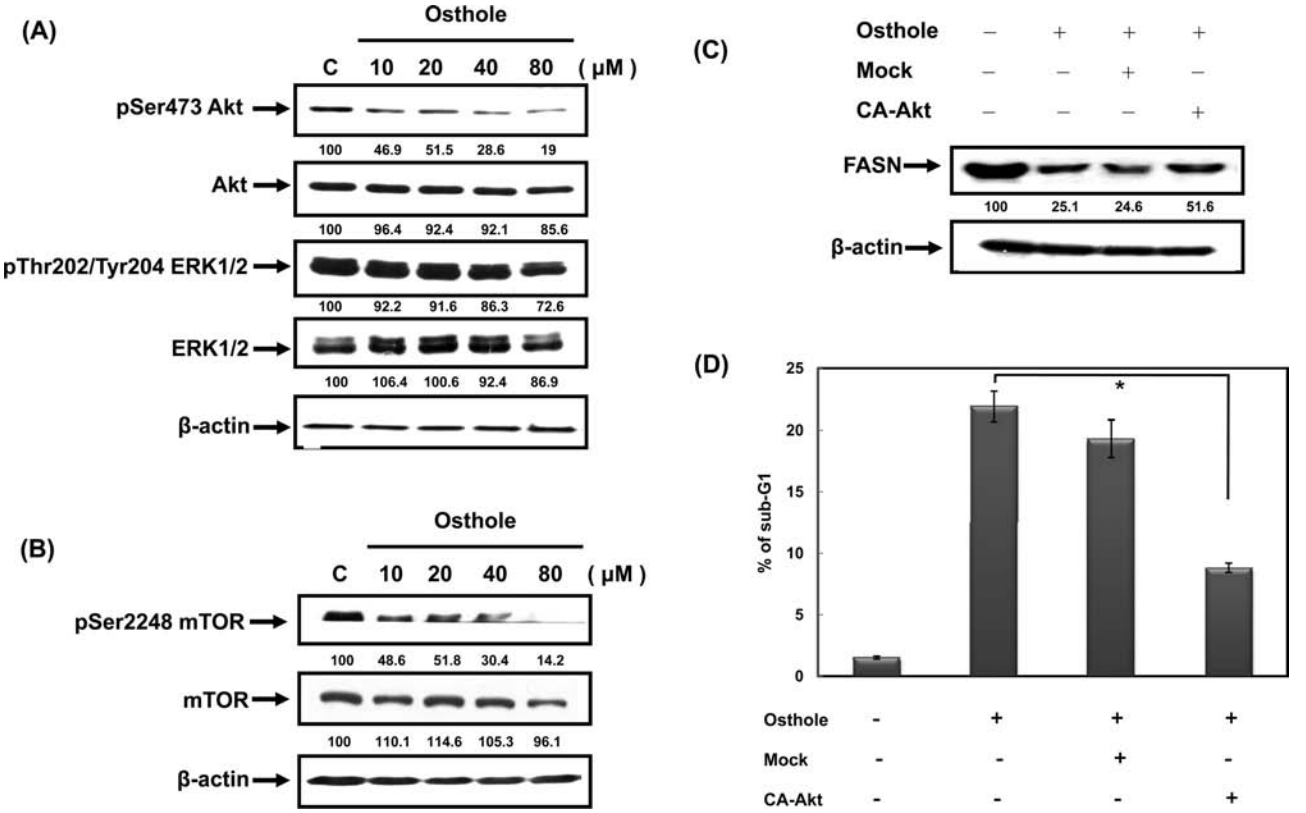

Figure 5. Effects of osthole on Akt, mTOR and MAP kinase phosphorylation in HER2-overexpressing cancer cells. SKOV3 cells were treated with various concentrations of osthole at 37 °C for 48 h. (**A**) Levels of phosphorylated Akt and ERK1/2 were analyzed by immunoblotting with phospho-Akt (Ser-473) and phospho-p44/42 MAPK (Thr202/Tyr204) antibodies. (**B**) Levels of phosphorylated mTOR were analyzed by immunoblotting with phospho-mTOR (Ser-2248) antibodies. (**C**) SKOV3 cells were transfected with 50 nmol/L CA-Akt or mock. Twenty-four hours after transfection, cells were treated with osthole (40 μM) for 48 h. Cells were harvested and immunoblotting was used to measure levels of FASN protein and β-actin. Immunoblotting with β-actin antibody demonstrated equivalent protein in each lane. Western blot data presented are representative of those obtained in at least three separate experiments. The values below the figures represent change in protein expression normalized to β-actin. (**D**) SKOV3 cells were transfected with 50 nmol/L CA-Akt or mock. Twenty-four hours after transfection, cells were treated with 40 μM osthole. After 48 h, cells harvested and subjected to sub-G1 DNA content analysis. The indicated percentages are the mean of three independent experiments. *Bars* represent the S.E. *, $p < 0.05$.

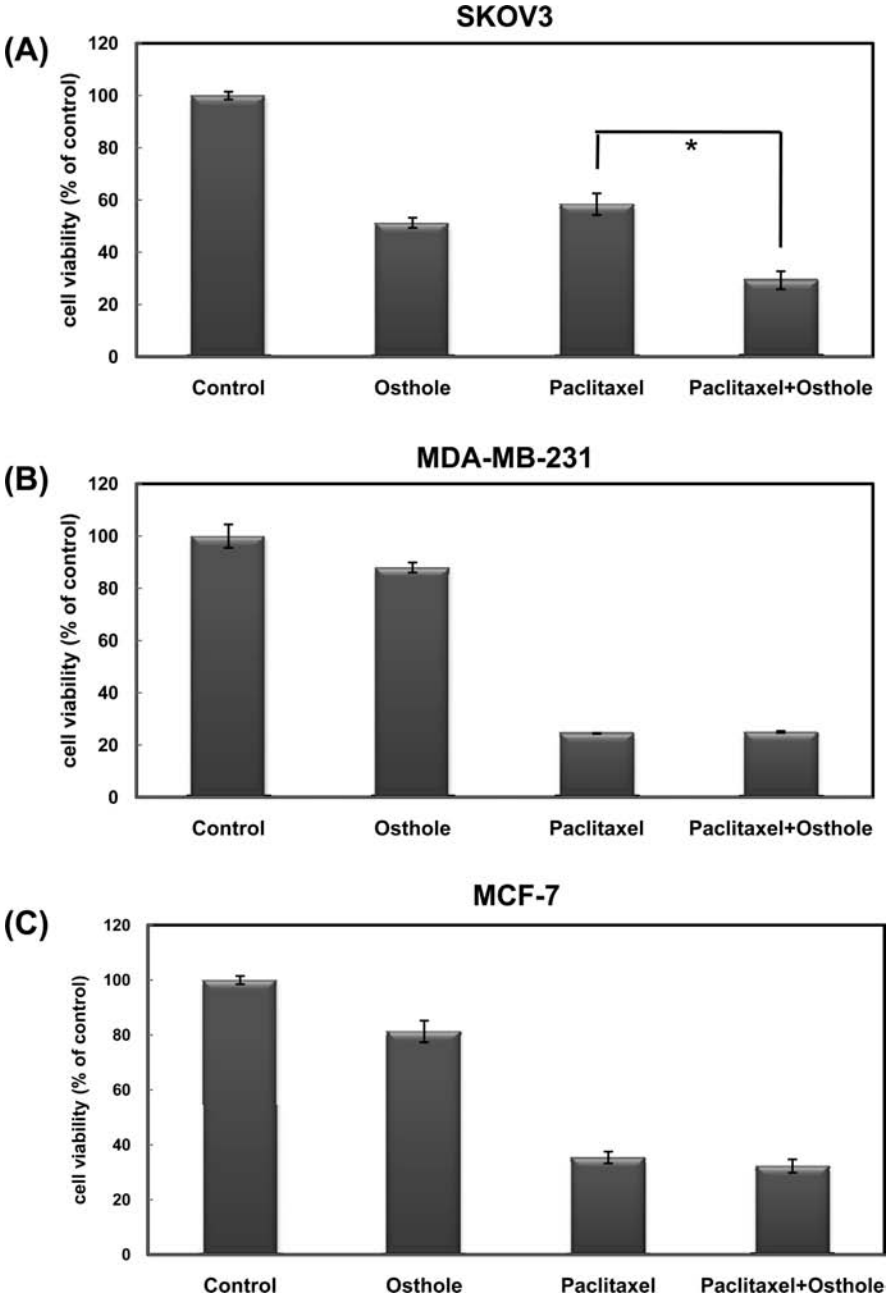

Figure 6. Osthole enhances paclitaxel-induced growth inhibition in HER2-overexpressing cancer cells. (**A**) SKOV3, (**B**) MDA-MB-231, and (**C**) MCF-7 cells were treated with osthole (40 μM) alone or in combination with paclitaxel (5 μM) at 37 °C for 48 h. The effects on cell growth were examined by the MTT assay, and the percentage of cell proliferation was calculated by defining the absorption of cells not treated with drugs as 100%. The number of viable cells after treatment is expressed as a percentage of the vehicle-only control. Data are means of three independent experiments. Bars represent the SE. *, $p < 0.05$.

option to cancer patients for centuries, whereas in Europe and North America, CAM has been growing in popularity only in the past few decades. In this study, we demonstrated that, among the tested naturally occurring constituents, osthole showed the most effective inhibition of FASN expression in HER2-overexpressing cancer cells. Osthole is an active constituent isolated from the fruit of *Cnidium monnieri* (L.) Cusson, a traditional Chinese medicine, and further analysis indicated that osthole preferentially inhibited the proliferation of HER2-overexpressing cancer cells. In addition, osthole could specifically sensitize HER2-overexpressing cancer cells to paclitaxel-induced cytotoxicity.

Osthole, a natural coumarin, is known to have a variety of pharmacological and biochemical uses and is considered to have potential antitumor activities. But the mechanism of cytotoxicity is still unclear. We demonstrated here for the first time that fatty acid synthesis is required for osthole-induced cytotoxicity in HER2-overexpressing cancer cells. Recent studies demonstrated that pharmacological inhibitors of FASN (including C75 and diosgenin) preferentially killed HER2-overexpressing breast epithelial cells, suggesting a link between FASN and carcinogenesis through its regulation of oncogenes (*24*). The opinion of oncogene addiction suggests that cancer cells are often physiologically dependent on the continued activity of specific activated or overexpressed oncogenes for maintenance of their malignant phenotype, which provides an Achilles' heel for tumors that can be exploited in cancer therapy (*25*). Moreover, most preinvasive breast cancer and premalignant breast lesions overexpress HER2; our data and others provide compelling evidence that FASN is a potential drug target for chemoprevention.

Recently, Kumar-Sinha et al. demonstrated that elevated FASN expression, which was driven by HER2 through PI3K/Akt signaling (*8*). Moreover, Wang et al. demonstrated that inhibition of FASN activity by C75 resulted in downregulation of phosphorylated Akt, which preceded the induction of apoptosis (*26*). In this study, osthole suppressed the phosphorylation of Akt in SKOV3 cells that overexpress HER2. Collectively, these data demonstrated that cancer cells with constitutively active Akt were protected from apoptosis, and that inhibition of the PI3K/Akt pathway increased their sensitivity to FASN inhibitor induced apoptosis, at least partially through Akt-regulated downregulation of FASN. Taken together, the downregulation of phosphorylated Akt by C75 inhibition of FASN *in vitro* is similar to our findings. These data suggest that FASN and fatty acid synthesis may play a role in oncogenesis through regulation of key oncogenic pathways on which cancer cells depend for survival and support FASN inhibition as a means to selectively kill cancer cells harboring HER2 overexpression.

Interestingly, our results illustrate that mTOR signaling plays an important role in the upregulation of FASN in HER2-overexpressing cancer cells, which is consistent with a recent report by Yoon et al. (*27*). There is a clear association between mTOR activation and HER2 overexpression in breast cancer (*28*), and activation of this signaling pathway has been associated with poorer prognosis. Many reports have suggested that the mTOR pathway plays an important role in the susceptibilities of chemopreventive or chemotherapeutic agents to kill cancer cells (*29*). Collectively, these data demonstrated that activation of the Akt/mTOR pathway has been found in cancers with high expression levels of HER2, suggesting possible roles of the Akt/mTOR pathway in HER2-mediated tumor progression.

Recent reports have pointed out that HER2-overexpressing cancer is resistant and able to relapse when treated with clinical drugs alone such as gefitinib and erlotinib (TKI), trastuzumab (EGFR mAbs), paclitaxel and cisplatin (mitotic inhibitor), and doxorubicin (topoisomerase II inhibitor). Thus, research has directed its inquiry toward the development of therapies specifically targeting HER2. The development of trastuzumab, a recombinant monoclonal antibody against HER2, initially proved to be a well-tolerated first line of treatment. However, in the long term patients, trastuzumab was shown to develop resistance to this monotherapy. Therefore, research on HER2 positive breast cancer has focused on the study of different anti-HER2 combination therapies over the past decade (*30*). In our present study, we found that osthole can preferentially inhibit HER2-overexpressing cancer cells. We also obtained synergistic efficacy on repressing proliferation of HER2-overexpressing cancer cells by combining paclitaxel *in vitro*. Our results provide a strategy for testing combinations of FASN inhibitors with other clinical drugs in clinical studies to achieve improved patient outcomes.

In conclusion, elevated FASN expression is associated with HER2 overexpression, which is regarded as a poor prognostic marker in breast cancer cells. We provide experimental evidence here for the first time to demonstrate that osthole could suppress FASN expression and modulate Akt and mTOR phosphorylation in HER2-overexpressing cancer cells. These results highlight the potential of use osthole as a unique chemotherapeutic modulator in the prevention or treatment of HER2-overexpressing cancer.

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