Cambogin Induces Caspase-Independent Apoptosis through the ROS/JNK Pathway and Epigenetic Regulation in Breast Cancer Cells

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Abstract

Cambogin is a polycyclic polyprenylated acylphoroglucinol (PPAP) from the Garcinia genus, which has been used traditionally for cancer treatment across Southeastern Asia. In this study, we found that cambogin inhibited breast cancer cell proliferation and induced cell apoptosis in vitro. Cambogin induced the activation of the caspase-independent mitochondrial apoptotic pathway, as indicated by an increase in the ratio of Bax/Bcl-2 and the nuclear translocation of apoptosis inducing factor (AIF). Two-dimensional gel electrophoresis and mass spectrometry revealed that the expression of proteins involving in the radical oxygen species (ROS) pathway was among the most affected upon cambogin treatment. Cambogin enhanced cellular ROS production, and induced the activation of the ASK1–MKK4/MKK7–JNK/SAPK signaling pathway. Pretreatment with ROS scavenger N-acetylcysteine (NAC), an antioxidant, or the JNK inhibitor SP600125 was able to restore cell viability in the presence of cambogin. Importantly, cambogin treatment led to the activation of activating transcription factor 2 (ATF-2) and the trimethylation of histone H3K9 in the activator protein 1 (AP-1) binding region of the Bcl-2 gene promoter. Finally, cambogin exhibited a potential antitumor effect in MCF-7 breast cancer xenografts without apparent toxicity. Taken in conjunction, the present study indicates that cambogin can induce breast adenocarcinoma cell apoptosis and therefore represents therapeutic potential for cancer treatment. Mol Cancer Ther; 14(7): 1738–49. ©2015 AACR.

Introduction

Breast cancer is the top ranked type of cancer and the second leading cause of cancer-related death in women worldwide in 2013 (1). In light of the recognition that obesity, lack of physical exercise, alcohol consumption, hormone replacement therapy during menopause, and ionizing radiation are mainly risk factors for breast cancer, it is envisaged that the incidence of breast cancer will remain high (2). Presently, mastectomy and chemotherapy provide the best prognosis for long-term survival, but unfortunately approximately 70% of patients are inoperable because of advanced tumor growth or bone metastasis (3). Therefore, there is an urgent need for identifying effective agents and developing new therapeutic strategies for the prevention and treatment of breast cancer.

natural products from herbal medicines have tremendous potential as promising drugs and remain a major source of drug discovery for decades (4). Polycyclic polyprenylated acylphoroglucinols (PPAP) are family of natural products that possess a characteristic bicyclo[3.3.1]nonane-2,4,9-trione core structure, decorated with prenyl or geranyl side chains and an additional acyl group (5). When the structural similarities between the PPAPs are considered, the broad spectrum of biologic activities exhibited by these compounds is particularly striking (6). For decades, PPAPs, the main bioactive components derived from Garcinia genus (Guttiferae family), have been subjected to extensive investigation as likely candidates for cancer treatment (7). For instance, Guttiferone K induced colon cancer G0-G1 cell cycle arrest and apoptosis as a result of p21Waf1/Cip1 upregulation (8). Oblongifolin C has antitumor effect via autophagic flux inhibition and apoptosis induction in cervical cancer (9, 10).

Our recent research has been focused on the discovery of novel and more selective PPAPs isolated from Garcinia genus in the treatment of breast cancer. Preliminary experiments indicated that cambogin, as a proapoptotic agent for breast cancer cells, is more potent compared with other PPAPs from Garcinia genus. Cambogin has more effective antitumor effect against breast cells than other compounds in the branches of Garcinia esculenta with high concentrations (11). Cambogin have been demonstrated to possess antitumor activity against medulloblastoma cells (12); however, the active component and the underlying mechanisms remain elusive.

Comparative proteomics, the large-scale study of proteins, can reveal novel diagnostic markers and potential drug targets through analyses of differential protein expression using two-
dimensional gel electrophoresis (2-DE) together with MALDI-MS (13). It is also a powerful approach to identify the potential molecular targets of traditional Chinese medicine on diseases (14). The present study performed 2-DE and MALDI-MS to characterize the proapoptotic properties of cambogin, and reveal that cambogin induced caspase-independent mitochondrial apoptosis via the ROS/JNK signaling pathway, the phosphorylation of activating transcription factor-2 (ATF-2) as well as trimethylation of histone H3K9 in the activator protein 1 (AP-1) binding region of the Bcl-2 gene promoter in breast cancer. Taken together, we provided the first evidence that the use of cambogin as a potential future treatment against breast cancer.

**Materials and Methods**

**Chemical compound**

Cambogin was isolated from the branches of *Garcinia esculenta*. Its structure was determined using $^1$H-NMR and $^{13}$C-NMR spectral analysis, and its purity was more than 98% as determined by high-pressure liquid chromatography analysis. Cambogin was dissolved in absolute DMSO as 100 mmol/L and, on the experimental day, was further diluted with culture medium.

**Cell lines and cell culture**

The human breast cancer cell lines (MCF-7, SK-BR-3, and MDA-MB-468), human cervical carcinoma cell line HeLa, human lung carcinoma cell line A549, human hepatic carcinoma cell line HepG2, and human colon carcinoma cell line HCT116 were purchased in 2013 to 2015 from the Chinese Academy of Science Collection Cell Bank (Shanghai, China). SK-BR-3, HeLa, and A549 cells were cultured in DMEM (Invitrogen) with 10% (v/v) FBS (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) at 37°C in a humidified atmosphere with 5% CO$_2$. HepG2 and HCT116 cells were cultured in Modified Eagle Medium (MEM, Invitrogen) with 10% (v/v) FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO$_2$. MCF-7 cells were cultured in DMEM with 10% (v/v) FBS, 0.01 mg/mL bovine insulin, and 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO$_2$. MDA-MB-468 cells were cultured in L15 medium (Invitrogen) with 10% (v/v) FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 0% CO$_2$. Authenticity of these cell lines was done by the authors.

**Cell viability assay**

After treatment, 3-(4,5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide (MTT) solution was added and incubated for 4 hours. After medium was removed, 100 μL DMSO was added and the absorbance was measured at 570 nm and cell viability was normalized as the percentage of control. For blocking study, cells were pretreated with 5 mmol/L N-acetylcysteine (NAC, Sigma), 20 μmol/L SP600125 (Cell Signaling Technology), 1,000 U/mL catalase (CAT, Sigma), or 100 U/mL superoxide dismutase (SOD, Sigma) for 30 minutes, and then treated with cambogin (10 μmol/L) for 24 hours.

**Cell-cycle distribution analysis**

After treatment, cells were harvested by trypsinization, washed twice with PBS, fixed with cold 70% ethanol overnight followed by staining with propidium iodide (PI) solution containing 50 μg/mL RNase A. The distribution of cell cycle was examined using FACSscan flow cytometer, and the data were analyzed by ModFit LT V3.0 software. The percentages of cell populations at Sub-G$_1$, G$_0$-G$_1$, S, and G$_2$–M phases were determined.

**Annexin V/PI staining**

After treatment, cells were harvested by trypsinization, washed twice with PBS, and stained with Annexin V-FITC/PI Cell Apoptosis Detection Kit (BD Biosciences) according to the manufacturer’s instructions. Apoptosis was quantified using FACSscan flow cytometer, and the data were analyzed with FACSField. The cells in the early stage of apoptosis were Annexin V positive and PI negative, whereas the cells in the late stage of apoptosis were both Annexin V and PI positive.

**Colony formation assay**

Four hundred cells were seeded per well in 6-well plates and cultured overnight. Cells were then treated with cambogin at various concentrations for 24 hours. After being rinsed with fresh medium, the cells were allowed to form colonies for indicated time periods, fixed, and then stained with 0.04% crystal violet. Colonies with more than 50 cells were counted as one positive colony under microscope. The inhibition of colony formation ratio was expressed as percentage of vehicle control.

**Hoechst 33342 staining assay**

After treatment, cells were incubated with Hoechst 33342 (10 μg/mL) for 5 minutes at room temperature in the dark. After incubation, stained cells were observed under a fluorescent microscope.

**Measurement of ROS formation**

Cells were stained with 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 minutes at 37°C in the dark, and then treated with vehicle alone (0.1% DMSO) or cambogin (10 μmol/L) for indicated time periods. After incubation, stained cells were observed under a fluorescent microscope and the absorbance was measured at 488 nm (excitation wavelength) and 525 nm (emission wavelength).

**Chromatin immunoprecipitation assay and real-time PCR (RT-PCR)**

After treatment, soluble chromatin was isolated using EZ-ChIP-Chromatin Immunoprecipitation kit (Millipore) as described by the manufacturer. The soluble chromatin was

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immunoprecipitated with anti-phospho-ATF-2 (Millipore) or anti-Histone H3R9me3 (abcam). PCR reaction was performed to amplify Bcl-2 promoter using chromatin immunoprecipitation (ChIP) primers (sense: 5'-GCTCGAGAACGTCCATGGA-3' and antisense: 5'-AATCGATAACGGAACGTCC-3'). The PCR products were done using a Power SYBR Green kit and performed on the ABI 7500 real-time PCR system.

**Two-dimensional electrophoresis (2-DE), protein visualization, and image analysis**

Cambogin or DMSO-treated MCF-7 cells were harvested and the protein concentration was determined using Bio-Rad Dc protein Assay. A 2-DE was performed as described (15).

**In vivo animal study**

Specific pathogen-free BALB/c nude female nude mice (7 weeks old) were purchased from the Experimental Animal Center of Chinese Academy of Science (Shanghai, China). The experimental procedures were approved by the Shanghai University of Traditional Chinese Medicine Committee on the Use of Live Animals for Teaching and Research and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, published by the NIH [publication No. SCXX(HU) 2007-0005]. MCF-7 breast cancer xenografts were performed as described (16). Nude mice (n = 6 each group) were given subcutaneous injection of MCF-7 cells (3 × 10^6 cells per mouse) into the mammary fat site. Before inoculation, 17β-estradiol (E2) pellet (1.7 mg, 60-day release, produce 0.3–0.4 nmol/L E2 Blood level; Innovative Research of America) was implanted subcutaneously followed by sealing of the incision with tissue adhesive Vetbond. After tumors had established (~50 mm³), nude mice were divided randomly into two groups. Solvent control (0.5% DMSO and 0.5% Tween-80 in normal saline) and cambogin (10 mg/kg in solvent control) were given via intraperitoneal injection every other day. After transplantation, the body weight and tumor sizes of all mice were recorded every 3 days. Tumor size was measured every other day. After transplantation, the body weight and tumor size of all mice were recorded every 3 days. Tumor size was measured every other day.

**Western blotting**

After treatment, cells were lysed in RIPA buffer containing 1 mmol/L PMSF and protease inhibitor cocktail. Lysates were spun at 10,000 g for 10 minutes and the supernatants were kept at −20°C until use. Performed as previously described (15). Please see Supplementary Table S1 for a complete list of antibodies used in this study.

**Immunofluorescence assay**

After treatment, cells were fixed with 4% PFA for 30 minutes at room temperature. Fixed cells were incubated with 0.3% Triton X-100 in PBS, and blocked with 10% BSA in PBS for 1 hour. The cells or tumor tissue sections (4 μm) were incubated with the primary antibody against AIF overnight at 4°C followed by Alexa-Fluor 488-conjugated goat anti-rabbit IgG antibody for 2 hours at room temperature. 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen) staining was then used to stain nuclei. Immunofluorescence images were visualized using an inverted fluorescent microscope (Olympus).

**Statistical analysis**

Data are presented as mean ± SEM. Statistical analysis was performed using one- or two-way ANOVA for multiple comparisons or Student unpaired t test for single comparisons. P values less than 0.05 were considered to indicate statistically significant differences.

**Results**

Cambogin inhibited proliferation and induced apoptosis in breast cancer cells

It has been reported in previous studies that traditional Chinese herb *Garcinia* genus possesses therapeutic effects in cancer treatments (8–13, 15). Therefore, we isolated several PPAPs from the *Garcinia* genus (7) and tested their effects on breast cancer cell proliferation (Fig. 1A), an important feature of cancer growth and development. One of those tested compounds, known as cambogin (chemical structure shown in Fig. 1B), markedly inhibited the proliferation of MCF-7 (human breast carcinoma). We also studied the cytotoxicity of cambogin in a panel of cancer cell lines using MTT assay. After cambogin (10 μmol/L) treatment for 48 hours, increased cytotoxicity was observed in all tested cell lines HeLa (human cervical carcinoma), A549 (human lung carcinoma), HepG2 (human hepatic carcinoma), HCT116 (human colon carcinoma), and MCF-7 (ER-PR-HER2+), SK-BR-3 (ER-PR-HER2+), and MDA-MB-468 (ER-PR-HER2+), also known as triple-negative breast cancer (TNBC; human breast carcinoma; Fig. 1C). Among them, breast cancer cell lines were the most sensitive to cambogin treatment. In contrast, cambogin did not affect the cell viability of HMEC-1 at the comparable dosage. As shown in Fig. 1D, MCF-7 cells exhibited time- and dose-dependent sensitivity of cambogin. Similarly, cambogin markedly inhibited the proliferation of MDA-MB-468 and SK-BR-3 cells in a time- and dose-dependent manner (Supplementary Fig. S1A and S1B). To study the potential effect of cambogin on long-term proliferation, we used colony formation assay. In MCF-7 (Fig. 1E), MDA-MB-468, and SK-BR-3 cells (Supplementary Fig. S1C and S1D), cambogin treatment suppressed colongenicity in a dose-dependent manner compared with the vehicle group.

To determine whether the reduction in cell proliferation by cambogin was resulted from the induction of apoptosis, we used Annexin V-FITC/PI double staining to quantitatively determine the apoptotic cells induced by cambogin in breast cancer cells. After the treatment with cambogin at 10 μmol/L for the indicated time periods, we observed an induction in the percentage of cells in Sub-G 1 phase with little change in the distribution of other cell-cycle phases in MCF-7 cells. Among them, breast cancer cell lines were the most sensitive to cambogin treatment. In contrast, cambogin did not affect the cell viability of HMEC-1 at the comparable dosage. As shown in Fig. 1D, MCF-7 cells exhibited time- and dose-dependent sensitivity of cambogin. Similarly, cambogin markedly inhibited the proliferation of MDA-MB-468 and SK-BR-3 cells in a time- and dose-dependent manner (Supplementary Fig. S1A and S1B). To study the potential effect of cambogin on long-term proliferation, we used colony formation assay. In MCF-7 (Fig. 1E), MDA-MB-468, and SK-BR-3 cells (Supplementary Fig. S1C and S1D), cambogin treatment suppressed colongenicity in a dose-dependent manner compared with the vehicle group.
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Figure 1.

VAD-FMK failed to restore cell viability in the presence of cam-caspase-9, and caspase-8 (Fig. 2C). Pan-caspase inhibitor Z-VAD-FMK failed to restore cell viability in the presence of cambogin (Fig. 2D), suggesting that cambogin induces caspase-independent apoptosis.

Since the translocation of AIF from the mitochondria to the nucleus has been considered as a critical event during the caspase-independent apoptosis (17), we asked whether cambogin induces the nuclear translocation of AIF in MCF-7 cells. Indeed, cambogin treatment led to the translocation of AIF from the mitochondria to the nucleus, as evidenced by Western blotting and immunofluorescence staining (Fig. 2E and 2F).

Cambogin induced the activation of the mitochondrial apoptotic pathway via AIF nuclear translocation in MCF-7 cells

To investigate whether cambogin induces apoptosis by triggering the mitochondrial apoptosis pathway, we examined the expression of the Bcl-2 family proteins, including Bcl-2, Bak, and Bax, in response to cambogin. We observed a decrease in Bcl-2 expression and an increase in the expression of Bak and Bax when cells were treated with cambogin, which led to an increase in the proapoptotic/antiapoptotic (Bax/Bcl-2) ratio in MCF-7 cells (Fig. 2A). MDA-MB-468, and SK-BR-3 cells (Supplementary Fig. S2A and S2B). The proapoptotic effect of cambogin in breast cancer cells was attenuated upon exogenously expressing Bcl-2 or knocking down Bax or Bak using siRNA in MCF-7 cells (Fig. 2B). MDA-MB-468, or SK-BR-3 cells (Supplementary Fig. S2C and S2D). However, there was little change in the expression of caspase-3, caspase-9, and caspase-8 (Fig. 2C). Pan-caspase inhibitor Z-VAD-FMK failed to restore cell viability in the presence of cambogin (Fig. 2D), suggesting that cambogin induces caspase-independent apoptosis.

Screening for differentially expressed proteins and protein network analysis revealed that cambogin induced the generation of ROS

To gain insight into how cambogin induces breast cancer cell apoptosis, we performed proteomics profiling of cambogin-treated cells by a comparative proteomics analysis. MALDI-TOF MS and MS/MS analyses identify 101 proteins (2-fold or more) either
significantly upregulated (49 proteins, 48.5%) or downregulated (52 proteins, 51.5%; Fig. 3A). Ingenuity pathway analysis (IPA) revealed that the affected proteins in response to cambogin are involved in multiple cellular pathways related to carcinogenesis, including cellular morphology, cellular proliferation and apoptosis, cellular movement, cellular metabolism, and cell to cell signaling and interaction, of which approximately 60% of the affected proteins fall into the class of "cellular proliferation and signaling and interaction, of which approximately 60% of the affected proteins fall into the class of "cellular proliferation and apoptosis" (Fig. 3B).

Notably, cambogin treatment led to a decrease in the protein expression of ANXA1, CTSD, HSPA9, HSPB1, LDHA, and TPI1, and an increase in the protein expression of ACTB, DLST, HSD17B10, and PPIA. These 10 proteins were directly involved in the generation of radical oxygen species (ROS), including synthesis, accumulation and production (Fig. 3C). Other 43 proteins indirectly involving in the ROS pathway were also affected upon cambogin treatment, according to the protein–protein interaction network built up with IPA database (Fig. 3D). Indeed, we observed an increase in ROS production after cambogin treatment (2.6-fold after 1 hour and 3.7-fold after 2 hours, respectively; Fig. 4A), and similar
Screening for changes in protein expression reveals that ROS network was affected by cambogin treatment. A, representative silver-stained-2-DE images of the MCF-7 cells treated with or without cambogin for 24 hours. Differentially expressed spots were shown by the arrows. B, Ingenuity pathway analysis (Ingenuity Systems, http://www.ingenuity.com). Bars represent molecular and cellular functions that were significantly changed following treatment with cambogin. C, changes in the expression levels of proteins directly involved in ROS generation in response to cambogin, based on IPA database. D, the protein–protein interaction network of ROS-related proteins based on IPA database was established.
effects were also observed in MDA-MB-468 and SK-BR-3 cells (Supplementary Fig. S3A and S3B). Similarly, cambogin induced an increase in the ratio of Bax/Bcl-2 and the nuclear translocation of AIF was attenuated if the cells were pretreated with ROS scavenger N-acetylcysteine (NAC; Fig. 4B and 4C). In parallel with this, the proportion of apoptotic cells was decreased from 85.8% to 10.9% in the presence of NAC in cambogin-treated cells (Fig. 4D). Likewise, cambogin-inhibited cell proliferation was abolished by NAC in MDA-MB-468 and SK-BR-3 cells (Supplementary Fig. S3C and S3D). We also studied whether cambogin has any effect on the expression of antioxidants such as GSH (reduced glutathione). GSSG (oxidized glutathione) levels were elevated upon cambogin treatment, whereas GSH levels were decreased from 4 to 24 hours, which indicates possible ROS production in cambogin-treated MCF-7 cells (Supplementary Fig. S4). Antioxidative enzymes, namely CAT and SOD, prevented the
inhibition of cell proliferation by cambogin treatment at 24 hours in MCF-7, MDA-MB-468, and SK-BR-3 cells (Supplementary Fig. S5).

JNK/SAPK signaling pathway was involved in cambogin-induced apoptotic responses

MAPK signaling pathways are composed of several subfamilies of kinases, including extracellular regulate protein kinase 1 and 2 (ERK1/2), p38 MAPK, and c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), all of which have been greatly implicated in controlling cell proliferation, differentiation, and apoptosis (18). In MCF-7 cells, JNK/SAPK was phosphorylated as early as 2 hours of incubation with cambogin, when reaching a maximal response at 8 hours, which persisted for 24 hours (Fig. 5A). In contrast, cambogin treatment only led to a modest inhibition of ERK1/2 phosphorylation at 16 and 24 hours, and little effect on p38 MAPK phosphorylation (Fig. 5A). We also studied the activation of protein kinases upstream of JNK/SAPK. Phosphorylation of ASK1 was increased after 2 hours of cambogin treatment. Furthermore, phosphorylation of MKK4 and MKK7 (downstream of ASK1) was also observed within 2 hours after cambogin stimulation, and was maintained for up to 24 hours (Fig. 5B). The activation of ASK1/MKK4 and MKK7/JNK could also be induced by cambogin in a time-dependent manner in MDA-MB-468 and SK-BR-3 cells (Supplementary Fig. S6A-S6D). Antioxidant NAC effectively antagonized the phosphorylation of JNK/SAPK in response to cambogin (Fig. 5C). Besides, JNK/SAPK-specific inhibitor SP600125 prevented cambogin-induced decrease in Bcl-2 protein expression, increase in Bax protein expression, and the nuclear translocation of AIF (Fig. 5D and 5E). Likewise, the proportion of apoptotic cells was decreased from 79.1% to 22.5% in the presence of SP600125 in cambogin-treated ones (Fig. 5F). This finding was confirmed in the MDA-MB-468 and SK-BR-3 cells (Supplementary Fig. S6E and S6F). These results suggest that the activation of JNK/SAPK is responsible for ROS-mediated cell apoptosis.

Cambogin decreased Bcl-2 expression by ATF-2–mediated epigenetic regulation

ATF-2, a component of AP-1 transcription factor, can be activated upon the stimulation of JNK/SAPK, which led to an increase in cell apoptosis in several types of cancers (19, 20).
Cambogin induced the binding of phospho-ATF-2 to the region of the Bcl-2 gene promoter, which may play an important role in inducing apoptosis of MCF-7 cells.

**Cambogin inhibited breast cancer growth and development in vivo**

To study the potential effect of cambogin on breast cancer growth and development, equal numbers of breast cancer cells were injected subcutaneously into the orthotopic mammary of nude mice. Once the tumors reached approximately 50 mm³, the mice were treated with i.p. injections of either the control (vehicle-treated) or cambogin (10 mg/kg) every other day. During the 35-day treatment, control tumors grew from 53.6 ± 6.0 mm³ to 779.0 ± 100.9 mm³, whereas cambogin-treated tumors grew from 52.1 ± 4.1 mm³ to 197.2 ± 36.7 mm³ (Fig. 7A and 7B). The data showed that tumor weight treated with cambogin at 10 mg/kg was reduced by 72.0% compared with the control group (Fig. 7C). There was no difference in body weight (Fig. 7D) and cell morphology of target organs, including brain, heart, lung, liver, spleen, and kidney (Fig. 7E) between the control and the cambogin-treated groups.

To investigate the mechanism by which cambogin inhibited tumor growth, we harvested the orthotopic mammary tumor from control- and cambogin-treated mice. We observed an increase in TUNEL-positive cells in response to cambogin in comparison with the control group (Fig. 7F). A decrease in Bcl-2 protein expression (Fig. 7G), an increase in Bax protein expression (Fig. 7G), the induction of JNK/SAPK phosphorylation (Fig. 7G), and AIF nuclear translocation (Fig. 7H) were also observed in the tumors of the cambogin-treated mice. Together, these results suggest that cambogin can inhibit breast cancer growth and development, at least in part via downregulation of Bcl-2 and the activation of JNK/SAPK, both in an AIF-dependent manner.

**Discussion**

Breast cancer is a complex and heterogeneous disease. Each breast cancer subtype, including ER, HER2, and TNBC, has different prognosis and treatment response (22). High mortality rates of mortality in breast cancer indicate that currently available treatments such as chemotherapy are still ineffective. *Garcinia* genus have been studied for more than 70 years and many bioactive compounds were identified with anticancer potentials; however, little is known about the active components involved and their biochemical mechanisms of action in breast cancer. In the present study, cambogin, a PPAP from the branches of *Garcinia* esculenta, was found to strongly inhibit cell proliferation and induce cell apoptosis in MCF-7 (ER⁺ PR⁺ HER2−), SK-BR-3 (ER⁻ PR⁻ HER2⁺), and MDA-MB-468 (ER⁻ PR⁻ HER2−, TNBC) cells, and to effectively suppress breast adenocarcinoma tumor growth without any apparent toxicity in vivo, indicating that cambogin is worthy for being developed into a therapeutic agent in the prevention and treatment of breast cancer.

Aptosis, an energy-dependent genetically programmed cell death, plays a pivotal role in the development of cancer, including cancer initiation, progression, and metastasis (23, 24). Apoptosis can be induced through either the extrinsic or the intrinsic pathway. The extrinsic pathway is triggered by death receptors (Fas, TNFR, and DR5) in response to ligand binding, leading to caspase-8 activation (25). The key event of the intrinsic pathway is the permeabilization of the mitochondrial outer membrane, which occurs in response to various stimuli, and is regulated by many cytoplasmic proteins including family members of Bcl-2. Bcl-2 is a thiol-containing protein that prevents apoptosis through the negative regulation of Bax and Bak mitochondrial translocation (27). The ratio of Bax/Bcl-2, as a candidate prognostic biomarkers for breast cancer, indicates the degree of mitochondrial outer membrane permeabilization and hence the entrance to the execution phase of the apoptotic program (28). The present study showed that a decrease in Bcl-2 expression accompanied by
Concomitant increases in Bax and Bak protein expression in response to cambogin. Caspase-dependent apoptosis is the major form of controlled cell death in cancer cells (29); however, cambogin did not activate the cleavage of caspase-3, caspase-9, and caspase-8. Of note, we found that cambogin triggered the nuclear translocation of AIF, which is a critical event for caspase-independent apoptosis (17). Accordingly, cambogin induces cell apoptosis through the caspase-independent mitochondria pathway.

Emerging evidence indicates that ROS generations are the byproducts of cellular oxidative processes and induce depolarization of the mitochondrial membrane, and consequently produce an increase in the levels of other proapoptotic molecules in the cells (30). High ROS levels are required for the initiation of apoptotic responses induced by several anticancer agents (31–33). The present findings provide direct evidence in the breast cancer cells that cambogin enhances ROS generation, which is responsible for the proapoptotic effects of cambogin on breast cancer cells. This is evidenced by the fact that ROS scavenger NAC is able to prevent most of these effects. Antioxidants, CAT and SOD, also prevent cambogin-induced cell death, suggesting a significant increase in O$_2^•$ and H$_2$O$_2$ generation in response to cambogin. O$_2^•$ is converted into water via the GSH peroxidase system (34). GSH is a ubiquitous reducing sulphydryl (-SH) tripeptide that participates in redox reactions by maintaining a reducing environment in the cells (35), which accounts for the depletion of GSH by cambogin in breast cancer cells. The decrease in GSH as well the increase in GSSG levels in response to cambogin may also account for the proapoptotic effect of cambogin in breast cancer cells.

MAPK pathways are one of the numerous downstream cascades of the ROS signaling pathway closely associated with cell proliferation, differentiation, mitosis, survival, and apoptosis (18, 36). The present study showed that cambogin treatment induced ROS-dependent activation of ASK-1, SEK1/MKK4, MKK7, and JNK/SAPK. The duration of JNK/SAPK activation regulates specific proapoptotic/antiapoptotic targets like Bcl-2 family of proteins, which is implicated in the apoptotic response when cells are exposed to several chemotherapeutic agents (33, 37, 38). Indeed, the blockade of JNK/SAPK activation attenuated the anticancer activity of cambogin, further supporting a central role of JNK/SAPK in mediating the proapoptotic effect of cambogin.

Figure 7. Cambogin inhibited breast adenocarcinoma growth and development in vivo. MCF-7 cells (3×10$^5$ cells per mouse) were injected subcutaneously into the orthotopic mammary of nude mice. Once the tumors reached approximately 50 mm$^3$, the mice were treated with i.p. injections of either the control (0.5% DMSO and 0.5% Tween-80 in normal saline) or cambogin (10 mg/kg) every other day. After the treatment for 35 days, the mice were killed. A, tumors were removed and photographed. Scale bar, 1 cm. B, tumor sizes were recorded every 3 days by Vernier caliper measurements and calculated as [(length×width$^2$)/2]. C, tumors were weighed. D, the body weight was recorded every 3 days. E, H&E staining sections in multiple organs in cambogin treatment group. Scale bar, 50 μm. F, tumor cell apoptosis in tumor sections from the cambogin-treated mice was confirmed by TUNEL-positive cells. Scale bar, 20 μm. G, Western blot analysis for the protein expression of phospho-JNK/SAPK, JNK/SAPK, Bcl-2, Bax, and GAPDH was measured in tumor sections of cambogin-treated mice. The left panel shows a representative immunoblot analysis and the right panel shows the data expressed as the ratio of Bax/Bcl-2. H, the translocation of AIF from mitochondria to nuclei was observed in tumor sections of cambogin-treated mice by immunofluorescence assay. Scale bar, 20 μm. Data are shown as mean ± SEM; *, P < 0.05; **, P < 0.01; ***, P < 0.001 compared with control. n = 6.
INK/SAPK and mitochondria metabolic pathways play crucial roles in metabolic diseases, inflammation, and many types of cancer, together with the data presented in this study, it can be tempting to speculate that in the development of future anti-breast cancer treatments, targeting these pathways is likely to be promising strategies.

ATF-2 is phosphorylated as a result of the activation of INK/SAPK or p38 MAPK, thereby promoting AP-1–dependent transcription of apoptotic genes, including Bcl-2 (19, 39). ATF-2 is known to regulate target genes by histone modification (20). It is also known that core histone methyltransferase methylcyatin can lead to gene silencing. Abnormal histone modification as well as DNA hypermethylation are usually correlated with epigenetic silencing of tumor suppressor genes, and subsequently lead to genomic instability (40, 41). Jenuwein and colleagues have demonstrated that in breast cancer cells, the methylation of H3K9, H3K27, and H4K20 is associated with transcription repression (42).

The present findings demonstrated that nuclear ATF-2 activation contributes to the apoptotic effect evoked by cambogin. The activation of ATF-2 subsequently led to an increase in histone H3K9 trimethylation in the AP-1–binding region of the Bcl-2 gene promoter, suggesting the importance of the epigenetic regulation involved in the proapoptotic effect of cambogin. In future studies, it is of utmost importance to unravel the molecular mechanisms by which the posttranslational modification of histone tails is disregulated in breast cancer cells.

In conclusion, our findings, from both in vitro and in vivo data, propose that cambogin functions as a proapoptotic agent through the ROS/INK/ATF-2/Bcl-2 signaling pathway and support the use of cambogin as a potential future treatment against breast cancer.

Our data also suggest that the development of therapeutic strategies targeting these pathways can be potentially beneficial in future battles against breast cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: K. Shen, J. Xie, H. Xu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Shen, H. Wang, F. Lu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Shen, J. Xie, M. Yu
Writing, review, and/or revision of the manuscript: K. Shen, J. Xie, H. Xu
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