BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 31(5), 526-535 (2023)

Cremastranone-Derived Homoisoflavanes Suppress the Growth of Breast Cancer Cells via Cell Cycle Arrest and Caspase-Independent Cell Death

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Abstract

Breast cancer is the most common cancer and a frequent cause of cancer-related deaths among women wordlwide. As therapeutic strategies for breast cancer have limitations, novel chemotherapeutic reagents and treatment strategies are needed. In this study, we investigated the anti-cancer effect of synthetic homoisoflavane derivatives of cremastranone on breast cancer cells. Homoisoflavane derivatives, SH-17059 and SH-19021, reduced cell proliferation through G2/M cell cycle arrest and induced caspase-independent cell death. These compounds increased heme oxygenase-1 (HO-1) and 5-aminolevulinic acid synthase 1 (ALAS1), suggesting downregulation of heme. They also induced reactive oxygen species (ROS) generation and lipid peroxidation. Furthermore, they reduced expression of glutathione peroxidase 4 (GPX4). Therefore, we suggest that the SH-17059 and SH-19021 induced the caspase-independent cell death through the accumulation of iron from heme degradation, and the ferroptosis might be one of the potential candidates for caspase-independent cell death.

Key Words: Breast cancer, Cremastranone, Homoisoflavane, Cell cycle arrest, Caspase-independent cell death, Anti-cancer

INTRODUCTION

Breast cancer is the most common malignant tumor and a frequent cause of cancer-related deaths among females globally (Sung *et al*., 2021). Breast cancer is classified into three major subtypes based on the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (Perou *et al*., 2000). Approximately 70% of breast cancer patients are hormone receptor positive/HER2 negative. HER2 positive cancer represents 15%-20% of the patients, and triple-negative breast cancer (TNBC) comprises 15% of the patients (Waks and Winer, 2019). The main treatment strategies for breast cancer are surgery, radiation, chemotherapy, endocrine therapy, and targeted therapy (Waks and Winer, 2019). Trastuzumab, a well-known therapeutic monoclonal antibody, is a representative agent to target HER2 for breast cancer patients (Hudis, 2007). Trastuzumab in combination with chemotherapy has led to significantly increased disease-free survival and overall survival of HER2 positive breast cancer patients (Slamon *et al*., 2011). For the treatment of hormone receptor-positive breast cancers, aromatase inhibitors, such as anastrozole and letrozole, in combination with cyclin-dependent kinase (CDK) 4/6 inhibitors are considered for the first-line therapy (Rossi *et al*., 2019; Waks and Winer, 2019). Despite these attempts in breast cancer treatment, metastasis and chemoresistance still remain as major problems in breast cancer treatment. In this context, further research is needed to study novel therapeutic targets to overcome the limitations of breast cancer treatment.

Natural products have been used as therapeutic agent for centuries and continued to be utilized for the treatment of various diseases to this day (Cragg and Pezzuto, 2016). Homoisoflavonoids (3-benzylidenechroman-4-ones), a type of phenolic compounds, are found mainly in plants, such as *Cae-*

Open Access **https://doi.org/10.4062/biomolther.2023.057**

Received Mar 14, 2023 **Revised** Apr 17, 2023 **Accepted** Apr 19, 2023
Published Online May 25, 2023

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salpinia sappan and *Ophiopogon japonicas* (Lin *et al*., 2014). Homoisoflavonoids are reported to have numerous biomedical properties, including anti-inflammatory (du Toit *et al*., 2005), antioxidative (Zhou *et al*., 2015), and wound healing activities (Rashed *et al*., 2003). For example, homoisoflavonoids from *Portulaca oleracea* have been shown to have cytotoxic activity in cancer cell lines (Yan *et al*., 2012) and cremastranone, a homoisoflavanone *from Cremastra appendiculata* to exhibit anti-angiogenic activity in endothelial cells (Shim *et al*., 2004; Kim *et al*., 2008). In addition, its synthetic derivatives were previously investigated for its anti-angiogenic potential *in vitro* and *in vivo* in ocular disease models (Lee *et al*., 2014; Basavarajappa *et al*., 2015). Furthermore, it has been reported that cremastranone-derived homoisoflavonoid is a protein-binding partner of ferrochelatase (FECH) and inhibits the activity of the FECH (Basavarajappa *et al*., 2017).

Programmed cell death, such as apoptosis, autophagy, and programmed necrosis, is mediated by cascades of intracellular events (Ouyang *et al*., 2012). In cancer cells, in contrast to normal cells, the apoptosis pathway is inhibited, and the phenomenon contributes to uncontrolled cell growth, metastasis, and resistance to anti-cancer therapies (Goldar *et al*., 2015; Pfeffer and Singh, 2018). Thus, targeting apoptosis is a promising strategy for anti-cancer therapy. Ferroptosis, a novel type of programmed cell death, was first proposed by Dixon in 2012 (Dixon *et al*., 2012). Ferroptosis is caused by increased lipid peroxidation via accumulation of iron or down-regulation of glutathione peroxidase 4 (GPX4). GPX4 is a major component in ferroptosis pathway, and GPX4 suppresses ferroptosis by acting as an antioxidant protein and is activated by glutathione (GSH) (Yu *et al*., 2021). To sustain abnormal growth, cancer cells exhibit an increased iron requirement compared with normal cells, and the characteristic can make cancer cells more susceptible to ferroptosis. Thus, ferroptosis has been attracting attention as a new target for cancer treatment. It is also considered as an alternative to overcome chemoresistance (Zhang *et al*., 2022).

Heme (iron-protoporphyrin IX) is an important cofactor involved in various biological processes. It acts as a prosthetic group in diverse hemoproteins, which participates in processes such as oxygen transport, oxygen storage, electron transfer, signal transduction, and metabolism of drugs and steroids. On the other hand, excess free heme causes oxidative stress, lipid peroxidation, and even cell death (Kumar and Bandyopadhyay, 2005). Therefore, intracellular heme homeostasis is regulated tightly by several defense mechanisms (Chiabrando *et al*., 2014). For example, 5-aminolevulinic acid synthase 1 (ALAS1) is a rate-limiting enzyme in the heme synthetic pathway and is negatively regulated by cellular heme (Ponka, 1997). Heme is degraded by heme oxygenase-1 (HO-1; HMOX-1) into biliverdin, ferrous iron, and carbon monoxide (CO) (Kikuchi *et al*., 2005). Recent studies show that dysregulation of heme metabolism seems to be associated with tumor progression (Wang *et al*., 2021). It indicates that targeting heme metabolism is a potent therapeutic strategy for the treatment of cancer.

In our previous study, we found that the synthetic homoisoflavane derivatives of cremastranone SH-17059, SH-19021, SH-19027 and SHA-035 decrease cell viability, and SH-19027 and SHA-035 induced cell cycle arrest and apoptosis in human and mouse colorectal cancer cells (Shin *et al*., 2022). In this study, we investigated the anti-cancer effects and action mechanisms of homoisoflavane derivatives, especially SH-17059 and SH-19021, in human breast cancer cells. We identified cell cycle arrest and caspase-independent cell death with some clues suggesting ferroptosis.

MATERIALS AND METHODS

Cell culture

T47D and ZR-75-1 (invasive breast carcinoma) cells were maintained in RPMI-1640 (Cytiva, Marlborough, MA, USA) with 10% fetal bovine serum (FBS, Cytiva) and 1X Antibiotic-Antimycotic (Biowest, Riverside, MO, USA) at 37℃ under a humidified atmosphere of 5% CO_2.

Antibodies and chemicals

Synthetic homoisoflavonoid derivatives of cremastranone (five homoisoflavanes SH-17059, SH-19021, SH-19026, SH-19027, and SHA-035, and a homoisoflavanone SH-19017; Fig. 1A) were produced as previously described (Shin *et al*., 2022). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The anti-c-Myc (#5606) monoclonal antibody and anti-CDK1 (#77055) polyclonal antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). The polyclonal antibodies anti-Cyclin D1 (sc-753), anti-p21 (sc-397), and anti-HO-1 (sc-136960) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The monoclonal antibodies anti-ALAS1 (ab154860) and anti-GPX4 (ab125066) were purchased from Abcam (Cambridge, UK).

Cell viability assay

T47D and ZR-75-1 cells (2.5×10^3 cells/well) were plated in 96-well culture plates and incubated overnight. Cell viability was measured with the water-soluble tetrazolium salt (WST)-based EZ-Cytox assay kit (DoGen Bio, Seoul, Korea). After treating the cells with SH-17059, SH-19017, SH-19021, SH-19026, SH-19027, or SHA-035 at the specified doses and durations, the WST solution was added to each well. After the plates were incubated for 2 h at 37°C, the cell viability was measured based on the absorbance at 450 nm using microplate reader (BioTek, Winooski, VT, USA).

Cell proliferation assay

A cell proliferation ELISA kit (Roche, Basel, Switzerland) was used to measure the cell proliferation rate according to the manufacturer's instructions. T47D and ZR-75-1 cells (2.5×10^3 cells/well) were plated in 96-well culture plates and incubated overnight. The T47D and ZR-75-1 were treated with SH-17059, SH-19021, SH-19027, or SHA-035 at the indicated doses for the indicated times, followed by labeling with BrdU labeling solution for 2 h at 37°C. After the cells were fixed with fixation solution, the cells were incubated with anti-BrdU antibody conjugated with peroxidase at room temperature for 90 min. After the cells were washed, the substrate solution was added, and then, the cells were incubated at room temperature for 30 min. Subsequently, H_2SO_4 was added to each well, and the absorbance at 450 nm with a reference wavelength of 690 nm was measured using a microplate reader (BioTek).

Western blot analysis

Harvested cells were lysed using lysis buffer (pH 8.0, 20 mM Tris-HCl, 10% glycerol, 137 mM NaCl, 10 mM EDTA,

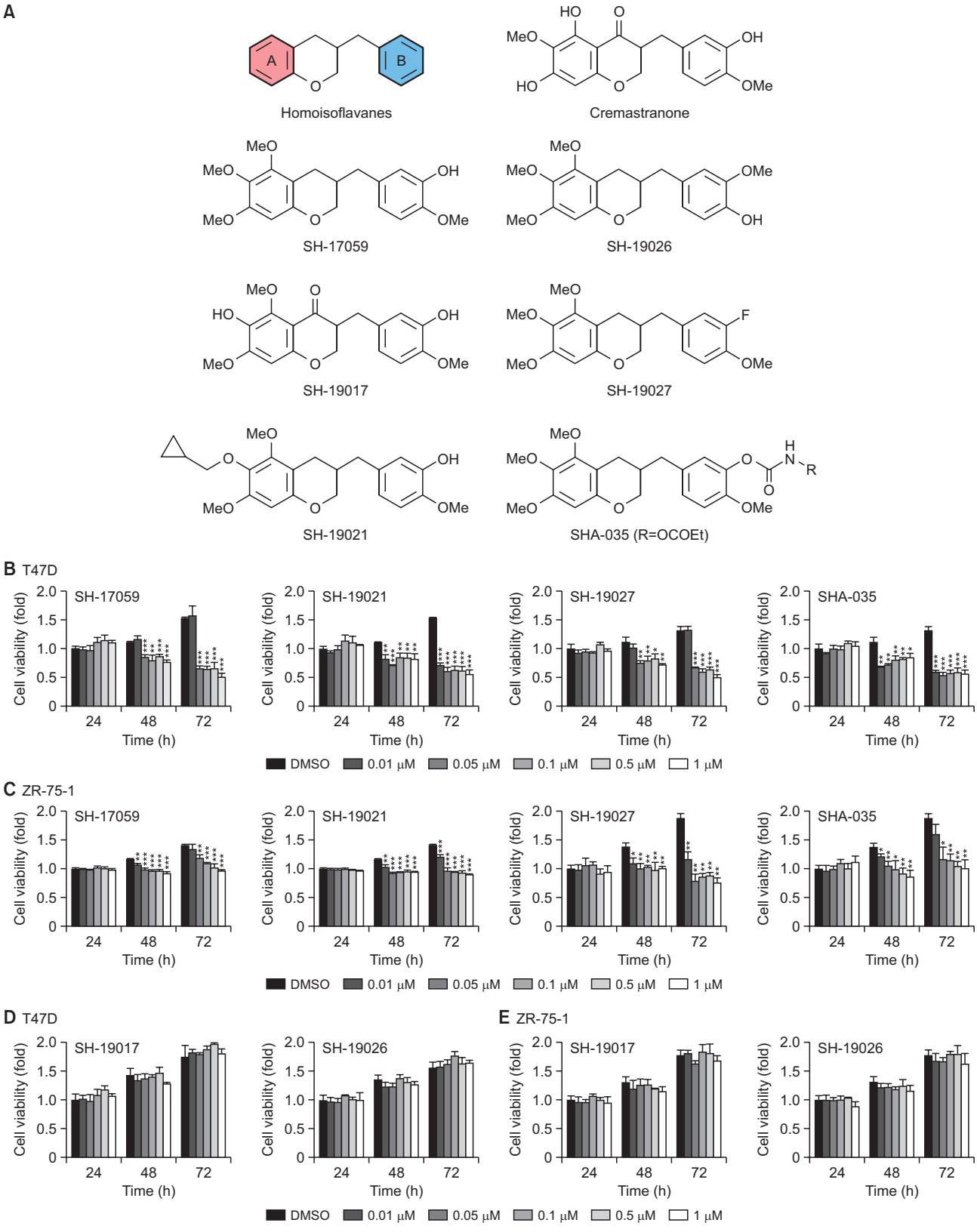

Fig. 1. The cytotoxic effect of homoisoflavane derivatives. (A) Structure of cremastranone and synthetic homoisoflavane derivatives (Shin et al., 2022). (B-E) T47D (B, D) and ZR-75-1 (C, E) cells were treated with the indicated doses of SH-17059, SH-17019, SH-19021, SH-19026, SH-19027, and SHA-035 for the indicated periods. The cell viability of the T47D and ZR-75-1 cells was measured using the WST assay. Values are the means ± SD. *p<0.05, **p<0.01, ***p<0.005 vs DMSO control. These are representatives of three independent experiments.

0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, phosphatase inhibitor, and protease inhibitor cocktail). Samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (Cytiva). The membranes were blocked with 5% skim milk in phosphate-buffered saline-Tween-20 (PBS-T; 140 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 2 mM KH_2PO_4, and 0.05% Tween-20). Proteins were immunoblotted with the appropriate primary antibody and then horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA). Immunoreactive proteins were detected with ECL solution (ATTO, Tokyo, Japan).

Cell cycle assay

After T47D cells were treated with the DMSO control, SH-17059, or SH-19021 for 48 h, fixation and permeabilization were performed using the BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences, Franklin Lakes, NJ, USA) for 20 min at 4°C. The cells were stained with propidium iodide (PI)/RNase staining buffer, and then, the fluorescent signal was detected by FACS Calibur (BD Biosciences). The cell cycle distribution was analyzed using the FCS Express program (De Novo Software, Glendale, CA, USA).

Apoptosis assay

Apoptosis was measured with the Annexin-V-FLOUS Staining Kit (Roche) according to the manufacturer's instructions. T47D cells were treated with DMSO, SH-17059, or SH-19021 for 48 h. The cells were stained with Annexin V-FITC and PI. The fluorescent signal was detected by FACSymphony A3 (BD Biosciences), and the data were analyzed using the FCS Express program.

Measurement of the HO-1 expression levels by FACS

For the detection of the intracellular HO-1 levels, T47D cells treated with SH-17059 or SH-19021 at the indicated dose for 48 h. The cells were fixed and permeabilized by the BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences) for 20 min at 4°C. The cells were incubated with anti-HO-1 (1:200) antibody for 1 h at 4°C. After washes, the cells were incubated with Alexa Fluor-conjugated goat anti-mouse IgG antibody (1:1,000; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 1 h at 4°C. The fluorescent signal was detected by NovoCyte® FACS (ACEA Biosciences, San Diego, CA, USA), and the data were analyzed using the FCS Express program.

ROS detection

2,7-dichlorodihydroflurescein diacetate (H_2DCF-DA) (Molecular Probes, Eugene, OR, USA) was used to measure the intracellular ROS levels. T47D cells were treated with SH-17059 or SH-19021 at the indicated dose for 48 h. After incubation with 5 μM of H_2DCF-DA for 30 min at 37°C, the fluorescent signal was detected by NovoCyte® FACS. The ROS level was analyzed using the FCS Express program.

Measurement of lipid peroxidation

BODIPY™ 581/591 C11 (Thermo Fisher Scientific, Inc.) was used to measure the lipid peroxidation levels. T47D cells were treated with SH-17059 and SH-19021 at the indicated dose for 48 h. The cells were stained with 2 μM BODIPY™ 581/591 C11 for 30 min at 37°C. The fluorescent signal was detected by NovoCyte® FACS. The lipid peroxidation levels were analyzed using the FCS Express program.

Statistics

The results represent the mean ± standard deviation (SD) from at least three independent experiments. Statistical significance of the differences between two sample groups was evaluated using Student's *t*-test. $p<0.05$ was considered statically significant.

RESULTS

The homoisoflavane derivatives have cytotoxicity in human breast cancer cells

To investigate the cytotoxic effect of the homoisoflavane derivatives in breast cancer cells, we measured cell viability of human breast cancer cells, T47D and ZR-75-1, after treatment with these compounds. The cell viability of both cell lines was reduced by the treatment with SH-17059, SH-19021, SH-19027, and SHA-035 in a time- and dose-dependent manner (Fig. 1B, 1C) with higher effect revealed in the T47D cells. In contrast, SH-19017 and SH-19026 had no or little effect on the T47D and ZR-75-1 cells (Fig. 1D, 1E), which is consistent with our previous results in colon cancer cells (Shin *et al*., 2022). These data indicate that SH-17059, SH-19021, SH-19027, and SHA-035 have a cytotoxic effect on human breast cancer cells.

The homoisoflavane derivatives decrease cell proliferation via inducing cell cycle arrest

Because the cell viability was reduced by these compounds, we measured the cell proliferation first. As shown in Fig. 2A and 2B, proliferation of the T47D and ZR-75-1 cells was significantly decreased by the homoisoflavane derivatives. Among the four active compounds, here, we focused on SH-17059 and SH-19021 and investigated their action mechanism using T47D cells for the next experiments. Because c-Myc and cyclin D1 are considered as proliferation markers (Motokura and Arnold, 1993; Dang, 2013), we measured their expression levels using western blot analysis. SH-17059 and SH-19021 decreased the expression level of c-Myc as expected (Fig. 2C left panel) but increased the expression level of cyclin D1 (Fig. 2C right panel).

Because SH-17059 and SH-19021 reduced cell proliferation, we next identified their effect on cell cycle by flow cytometry analysis. SH-17059 and SH-19021 increased the cell population in the G2/M phase compared to the DMSO control (Fig. 2D). CDK1 has a role as an inducer of G2/M phase progression, and p21 inhibits the expression and activity of CDK1 (Vermeulen *et al*., 2003; Matthews *et al*., 2022). Thus, we analyzed the expression levels of CDK1 and p21 as G2/M phase protein markers using western blot analysis. The CDK1 expression was reduced whereas p21 expression was increased by the SH-17059 and SH-19021 treatment (Fig. 2E). Therefore, these results suggest that the cell cycle arrest induced by SH-17059 and SH-19021 contributes to the reduced viability and proliferation in human breast cancer cells.

SH-17059 and SH-19021 induced caspase-independent cell death in T47D cells

In our previous study, we identified that homoisoflavane derivatives induced apoptosis in colorectal cancer cells (Shin

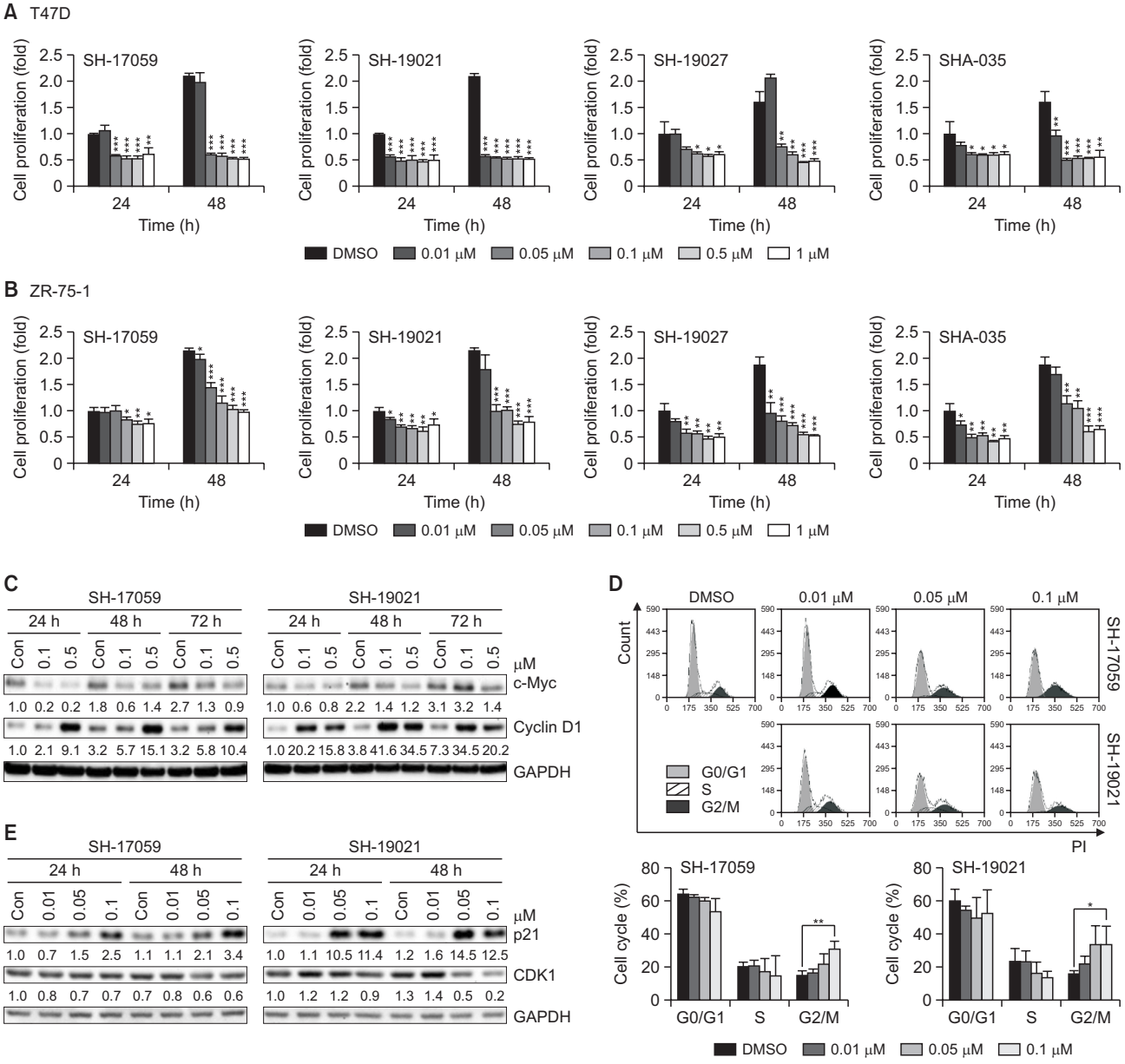

Fig. 2. The homoisoflavane derivatives reduce cell proliferation via cell cycle arrest at the G2/M phase. (A, B) T47D (A) and ZR-75-1 (B) cells were treated with SH-17059, SH-19021, SH-19027, and SHA-035 for the indicated periods, and then BrdU incorporation assay was performed. (C, E) The T47D cells were treated with the indicated doses of SH-17059 and SH-19021, and the cell lysates were subjected to western blot analysis. GAPDH was used as a loading control. (D) The T47D cells were obtained after treatment with the indicated dose of SH-17059 and SH-19021 for 48 h. The cell cycle was analyzed by flow cytometry after PI staining. The distribution of cells in the G0/G1, S, and G2/M phases of the cell cycle are presented. Values are the means ± SD. *p<0.05, **p<0.01, ***p<0.005 vs DMSO controls. These are representatives of three independent experiments.

et al., 2022). In apoptotic cells, the exposure of phosphatidylserine (PS) on the outer plasma membrane is a main feature, which result in phagocytosis (Fadok *et al*., 1992). As shown in Fig. 3A, treatment of T47D cells with SH-17059 or SH-19021 significantly increased annexin V-positive cells compared to the DMSO controls. Because apoptosis is initiated by the activation of caspases (Van Cruchten and Van Den Broeck, 2002), we investigated caspase 3, 8, 9 and 10 activation after treatment with SH-17059 or SH-19021. However, there was no change in the caspase activity (Fig. 3B). In addition, pretreatment with Z-VAD-FMK, a pan-caspase inhibitor, did not affect the cell viability (Fig. 3C). Taken together, these results indicate that SH-17059 and SH-19021 induced the cell death of breast cancer cells, but it was not a caspase-dependent apoptosis.

Heme was downregulated by SH-17059 and SH-19021 in T47D cells

Cremastranone-derived homoisoflavonoids were previously reported to bind with FECH and inhibit its activity (Basavarajappa *et al*., 2017). FECH, also known as heme synthase, is a terminal enzyme of heme synthesis. Thus, we hypothesized that SH-17059 and SH-19021 might reduce heme synthesis. To investigate the hypothesis, we checked the expression of the heme related proteins, ALAS1 and HO-1. As shown in the Fig. 4A and 4B, the expression levels of ALAS1 and HO-1 proteins were increased in the cells treated with SH-17059 or SH-19021. ALAS1 is a key enzyme of heme biosynthesis and

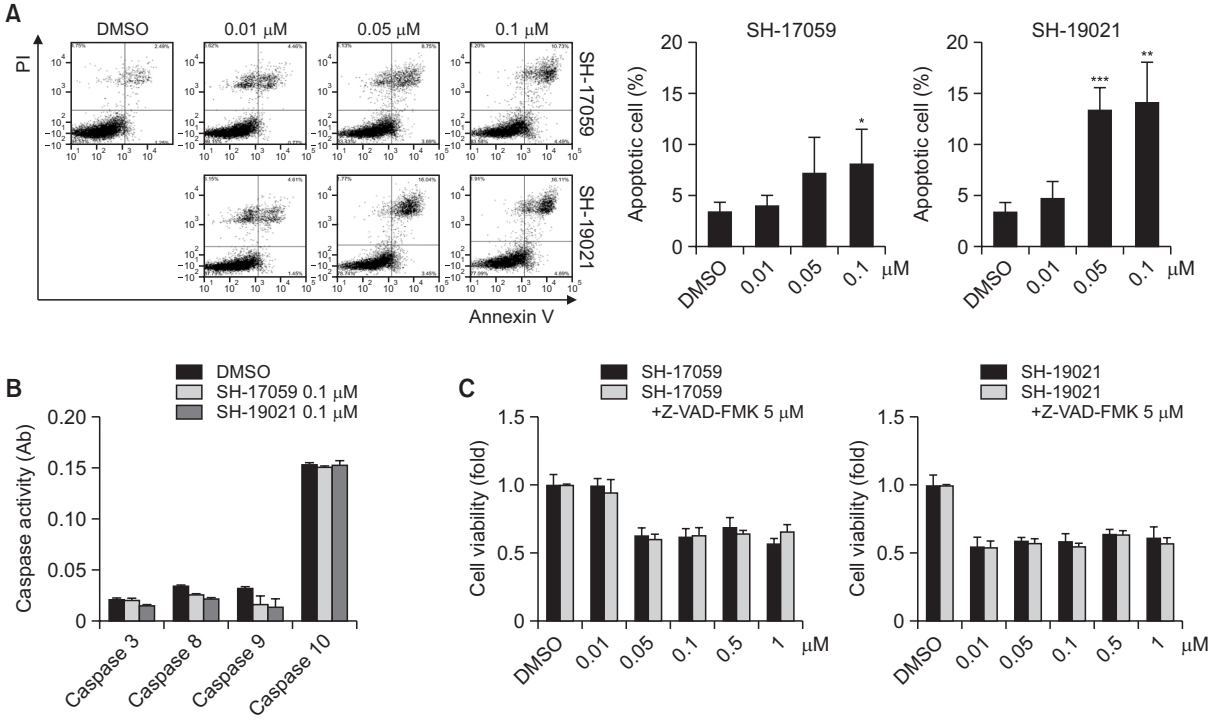

Fig. 3. SH-17059 and SH-19021 induce caspase-independent cell death. (A) The T47D cells were obtained after treatment with the indicated doses of SH-17059 and SH-19021 for 48 h. The percentages of apoptotic cells were detected by flow cytometry after annexin V-FITC and PI staining. (B) T47D cells were treated with 0.1 μM of SH-17059 or SH-19021 for 48 h. The caspase activity was measured using each colorimetric caspase substrate. (C) The T47D cells were untreated or pre-treated with Z-VAD-FMK for 1 h followed by treatment with the indicated doses of SH-17059 and SH-19021 for 72 h. The cell viability of the T47D cells was measured using the WST assay. Cell viability was normalized with each DMSO control. Values are the means ± SD. *p<0.05, **p<0.01, ***p<0.005 vs DMSO controls. These are representatives of three independent experiments.

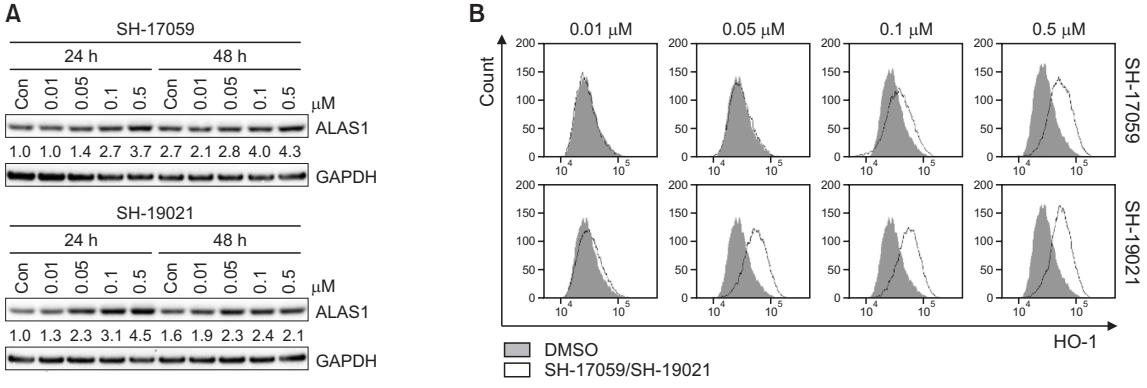

Fig. 4. SH-17059 and SH-19021 upregulate the expression levels of HO-1 and ALAS1. (A) The T47D cells were treated with the indicated doses of SH-17059 and SH-19021 for the indicated times. (A) The protein of ALAS1 was detected by western blot analysis. The relative expression of ALAS1 was indicated after normalization with the value of DMSO control at 24 h. (B) The T47D cells were treated with the indicated doses of SH-17059 and SH-19021 for 48 h. The HO-1 expression level was analyzed by flow cytometry. These are representatives of three independent experiments.

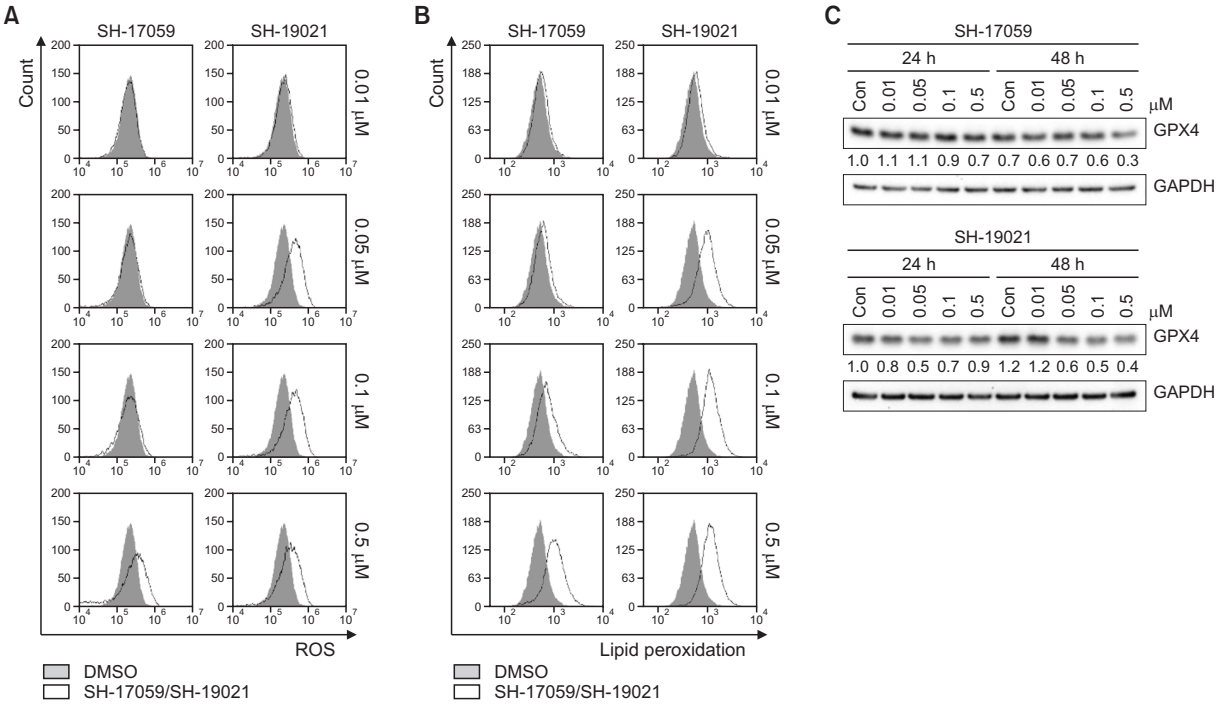

Fig. 5. SH-17059 and SH-19021 induce the ROS generation, lipid peroxidation, and GPX4 reduction. (A, B) The T47D cells were treated with SH-17059 or SH-19021 at the indicated doses for 48 h. Intracellular ROS levels were detected by flow cytometry using H_2DCF-DA (A). The levels of lipid peroxidation were analyzed using flow cytometry and BODIPY™ 581/591 C11 (B). (C) The T47D cells were treated with the indicated doses of SH-17059 and SH-19021 for 24 h or 48 h, and the cell lysates were subjected to western blot analysis. The relative expression of GPX4 was indicated after normalization with the value of each DMSO control at 24 h. These are representatives of three independent experiments.

regulated by feedback inhibition (Ponka, 1997); therefore, its induction implies the downregulation of heme. HO-1 is known to degrade heme and induced by various phytochemicals including resveratrol and flavonoids (Kikuchi *et al*., 2005; Ferrándiz and Devesa, 2008). Therefore, these results suggest that SH-17059 and SH-19021 induce the downregulation of heme, and the increased HO-1 expression may result in the degradation of heme.

SH-17059 and SH-19021 induced an increase of ROS and lipid peroxidation in T47D cells

HO-1 catalyzes heme to biliverdin, CO, and ferrous iron. Because iron accumulation may lead to the generation of ROS through the Fenton reaction (Dix and Aikens, 1993), we first measured the intracellular ROS levels using an intracellular ROS sensor, H_2DCF-DA. As shown in Fig. 5A, the ROS levels were increased by SH-17059 and SH-19021 at concentrations higher than 0.05 μM. As iron accumulation and iron-mediated ROS generation may induce ferroptosis (Dixon *et al*., 2012), we detected the levels of lipid peroxidation, a ferroptosis marker, using a lipid peroxidation sensor, BODIPY™ 581/591 C11. The lipid peroxidation levels were increased in the cells treated with SH-17059 or SH-19021 (Fig. 5B). It has been reported that iron overload decreases GPX4 expression and induces ferroptosis through p53-mediated transcriptional repression of the cystine/glutamate antiporter SLC7A11 (Huang *et al*., 2021). Therefore, we measured the expression levels of GPX4 as a ferroptosis marker and found that SH-17059 and SH-19021 decreased the expression level of GPX4 in a dose-dependent manner (Fig. 5C). Taken together, these results suggest a possibility that SH-17059 and SH-19021 induces ferroptosis in breast cancer cells.

DISCUSSION

Advances in breast cancer treatment have increased the survival rates of breast cancer patients over the past decades. However, research on novel chemotherapeutic reagents and novel treatment strategies is still needed. Natural homoisoflavanone compounds have been isolated from a variety of plants (Lin *et al*., 2014). Cremastranone, one of the homoisoflavanones, and its synthetic derivatives have been reported to have anti-proliferative and anti-angiogenic activity in endothelial cells (Shim *et al*., 2004; Lee *et al*., 2014; Basavarajappa *et al*., 2015). In this study, we demonstrated that synthetic homoisoflavane derivatives of cremastranone have cytotoxicity in human breast cancer cells. According to previously reported studies, other homoisoflavonoids had cytotoxicity against various cancer cell lines including breast cancer cells with IC_{50} in the micromolar range (Nguyen *et al*., 2006; Yan *et al*., 2012; Zhou *et al*., 2013). However, homoisoflavane derivatives of cremastranone that we used in this study exhibited cytotoxic effects at nanomolar concentrations in human breast cancer cells. Therefore, these compounds could be used as effective anticancer agents by reducing side effects.

As we previously reported, four synthetic homoisoflavanes (SH-19027, SHA-035, SH-17059 and SH-19021) exerted an

anti-cancer effect in colorectal cancer cells. SH-19027 and SHA-035 were reported to possess anti-cancer activity associated with cell cycle arrest and apoptosis in colon cancer cells (Shin *et al*., 2022). Here, we found that the four compounds have an anti-cancer effect in breast cancer cells as well; however, there was partial difference in the mechanisms. Based on the detailed study, the other two compounds, SH-17059 and SH-19021, also induced cell cycle arrest and increased the annexin V-positive cell population; however, the cell death was caspase-independent, which is different from general apoptosis. Caspase-independent cell death occurs in some cell death models, such as ferroptosis, parthanatos, lysosome-dependent cell death, and autophagic cell death (Fitzwalter and Thorburn, 2015; Galluzzi *et al*., 2018). Previously, it was reported that RSL3 (a GPX4 inhibitor)-induced ferroptosis is also accompanied by an annexin V-positive cell population (Sui *et al*., 2018). Together with other evidence, we conclude that the type of cell death was caspase-independent cell death like ferroptosis rather than caspase-dependent apoptosis in breast cancer cells. Therefore, homoisoflavane derivatives may be used generally in various cancers even though the action mechanisms might be partly different depending on the cell types. Whether homoisoflavane derivatives induce caspase-independent cell death also in colon cancer cells and whether there are differences in the action mechanisms among the four potent homoisoflavane derivatives need further study.

We observed that homoisoflavane derivatives commonly increased the expression of cyclin D1 in colon cancer cells (Shin *et al*., 2022) and breast cancer cells. Although cyclin D1 is considered as a cell proliferation marker, it is likely that the upregulation of cyclin D1 contributes to the increased sensitivity of the cells to cell death. Ectopic overexpression of cyclin D1 induced more apoptosis in breast cancer cells treated with the proteasome inhibitor bortezomib (Ishii *et al*., 2006). Overexpression of cyclin D1 increased sensitivity to fenretinide-induced apoptosis in breast cancer cells (Pirkmaier *et al*., 2003). Additionally, overexpression of cyclin D1 induced apoptosis in the neural cell line N1E-115, and cyclin D1-dependent kinase was activated during the neuronal apoptosis (Kranenburg *et al*., 1996). Therefore, the functional role of cyclin D1 in the cell death induced by these compounds is another issue to pursue.

In breast cancer cells, SH-17059 and SH-19021 increased the HO-1 expression which agrees with previous reports that HO-1 is induced by several stimuli such as oxidative stress, free heme, and phytochemicals including flavonoids (Kikuchi *et al*., 2005; Ferrándiz and Devesa, 2008). Although HO-1 is expressed in diverse cancers and related to a poor prognosis and immune suppression (Luu Hoang *et al*., 2021), HO-1 has been reported to induce ferroptosis through iron accumulation in recent studies (Chiang *et al*., 2018). Furthermore, it was reported that a HO-1 knockdown alleviated the ferroptosis induced by S-dimethylarsino-glutathione (ZIO-101; Darinaparsin) treatment in leukemia cells (Xu *et al*., 2022). Therefore, increased expression of HO-1 induced by SH-17059 and SH-19021 is presumed to increase the heme degradation and iron release. Considering that ALAS1 is regulated by feedback inhibition (Ponka, 1997), induction of ALAS1 further supports heme downregulation. In turn, SH-17059 and SH-19021 induced the generation of reactive oxygen species (ROS) and lipid peroxidation, while also decreasing GPX4 expression. These findings support our hypothesis that these compounds may initiate ferroptosis, which could potentially contribute to the anti-cancer effects observed in breast cancer cells, at least partially. The mechanism and functional significance of HO-1 induction in the cremastranone derivative-induced cell death have to be further investigated.

In summary, we investigated the anti-cancer effect of synthetic homoisoflavane derivatives of cremastranone in human breast cancer cells. Treatment with these compounds decreased the cell viability accompanying G2/M phase cell cycle arrest and caspase-independent cell death along with ROS generation and lipid peroxidation. We believe that these data will contribute to the development of novel strategies for cancer therapy against breast cancer.

ACKNOWLEDGMENTS

This work was supported by grants from the National Research Foundation funded by the Korean government, Ministry of Science, ICT, and Future Planning (NRF-2020R1I-1A1A01072992, NRF-2021R1A2C1006767).

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