Yuanhuatine from Daphne genkwa selectively induces mitochondrial apoptosis in estrogen receptor α -positive breast cancer cells in vitro

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ABSTRACT

Breast cancer is one of the most common cancers diagnosed among women worldwide. Estrogen receptor alpha (ER α) is a transcriptional factor that plays an important role in the development and progression of breast cancer. Yuanhuatine, a natural daphnane-type diterpenoid extracted from Daphne genkwa, was reported to exhibit significant cytotoxicity against breast cancer cells. However, the underlying mechanism is still unclear. In this study, we evaluated the cytotoxicity of yuanhuatine on two breast cancer cell lines that are ERαpositive and -negative. The results show that yuanhuatine inhibits the growth of $ER\alpha$ -positive cells (MCF-7) with much stronger inhibitory activity (IC₅₀ = $0.62 \,\mu$ M) compared with positive control tamoxifen (IC₅₀ = $14.43 \,\mu$ M). However, no obvious cytotoxicity was observed in ERa-negative cells (MDA-MB-231). Subsequent experiment also indicated that yuanhuatine markedly induced mitochondrial dysfunction, leading to apoptosis in MCF-7 cells. Molecular docking studies suggest the potential interactions between yuanhuatine and ERα. Immunofluorescence staining and Western blot analysis indicated that yuanhuatine down-regulated the expression of ER α in MCF-7 cells. MPP, a specific ER α inhibitor, significantly enhanced yuanhuatine-induced mitochondrial dysfunction and apoptosis in MCF-7 cells. On the contrary, the treatment with yuanhuatine causes no apoptosis in MM231 cells. Altogether, in vitro and in silico results suggested that ERa downregulation was involved in yuanhuatine-induced mitochondrial dysfunction and apoptosis in ERa-positive breast cancer cells. Thus, yuanhuatine could be a potential candidate for treating ER α -positive breast cancer.

ABBREVIATIONS

ERα	estrogen receptor alpha
ERK	extracellular signal-regulated kinase
MEK	mitogen-activated protein kinase kinase
MM231	MDA-MB-231
MMP	mitochondrial membrane potential
mTOR	mammalian target of rapamycin
ROS	reactive oxygen species

Introduction

Breast cancer is currently one of the most prevalent cancers and the leading cause of cancer-related deaths among women worldwide [1]. The pathogenesis of breast cancer has been considered to be associated with hormone stimulation of estrogen receptor alpha (ER α) [2]. It has been known that ER α is overexpressed in approximately 65% of human breast cancers [3], which is critical for estrogen-dependent growth. Therefore, the diagnosis of breast cancer falls into two broad categories (ER α -positive or ER α -negative) based on the presence or absence of ER α in cancer cells. Despite advances in diagnosis and target-treatment in ER α positive breast cancer, a poor outcome still remains [4, 5]. Thus, more effective and selective agents are required for the treatment of ER α -positive breast cancer.

Recently, interest in natural compounds as anticancer agents has grown, in the aspects of long term use and safety. As a wellknown traditional A Chinese medicine, *Daphne genkwa*, from the dried flower buds of *Daphne genkwa* Sieb. et Zucc. (Thymelaeaceae), has been used for diuretic, antitussive, abortifacient, and antitumor efficacy in the past centuries [6]. Previous phytochemical studies on the chemical components in the flower buds of this plant have identified a series of constituents, including daphnanetype diterpenoids [7]. Daphnane-type diterpenoids are typical biologically active constituents existing predominantly in *Daphne genkwa*, which exhibited excellent inhibitory activities against a variety of human cancer cells [8–10].

In this study, yuanhuatine (**> Fig. 1A**), a daphnane-type diterpenoid, isolated in our lab from an ethanol extract of the flower buds of *Daphne genkwa* as previously described [11], is shown to exhibit significant growth inhibitory effects. The effect on ER α -positive breast cancer cell lines is far stronger than that of tamoxifen, a well-known drug. The primary objective of this study is to examine the growth inhibitory effects of yuanhuatine on human breast cancer cells *in vitro* and to elucidate the underlying molecular mechanisms.

Results

Our previous study evaluated the inhibitory effects of yuanhuatine on 10 selected human cancer cell lines [11]. The results showed that yuanhuatine displayed extensive inhibitory effects in most cancer cell lines, especially in MCF-7. Therefore, two breast cancer cell lines (MCF-7 and MM231), as well as human normal epithelial cells (MCF10A) were further investigated by sulforhodamine B (SRB) assay for the inhibitory effects of yuanhuatine $(0-100 \ \mu\text{M})$ after 48 h treatment of the cells. As shown in **Fig. 1B**, yuanhuatine exhibited significant inhibitory effect on MCF-7 cells (ER α -positive) with an IC₅₀ of 0.62 μ M, less inhibitory effect in MM231 (ER α -negative) cells, and no obvious toxic effect in normal MCF10A. It's worth mentioning that yuanhuatine exhibited even more potent inhibitory capacity than tamoxifen which was used as a positive control (**Fig. 1C**).

These results indicate that yuanhuatine possesses a potent capacity of repressing ER α -positive cells (MCF-7) more selectively than ER α -negative cells (MM231).

The results in **Fig. 1** showing the selectively growth inhibitory effect of yuanhuatine motivated us to further investigate the underlying mechanisms in MCF-7 cells. Apoptotic cells undergo characteristic changes in cell morphology, including rounding, shrinkage and membrane blebbing [12]. Such morphological changes were observed in yuanhuatine-treated MCF-7 cells by phase contrast microscopy (> Fig. 2A). Meanwhile, nuclear chromatin condensation and fragmentation were examined by Hoechst 33 258 staining in MCF-7 cells after treatment with yuanhuatine (> Fig. 2B). The induction of apoptosis in MCF-7 cells was further analyzed by flow cytometry (> Fig. 2C). We further determined the protein expression of the key executors of cell apoptosis, PARP and caspase-7 [13]. Western blot analysis showed that treatment of yuanhuatine decreased the levels of pro-caspase-7 and PARP, and increased the level of cleaved caspase-7 (> Fig. 2 D, E).

Cell viability increased when cells were co-treated with yuanhuatine and Z-VAD-fmk, a pan caspase inhibitor [14], (20 μ M), where the percentages of apoptotic cells decreased markedly (**> Fig. 2F, G**). These data indicate that yuanhuatine induces apoptosis through a caspase-mediated pathway in MCF-7 cells.

Mitochondria play a central role in regulating apoptosis. It has been reported that the decrease of mitochondrial membrane potential (MMP) and subsequent release of reactive oxygen species (ROS) induce cell death via activating mitochondrial pathway of apoptosis [15, 16]. Rhodamine 123 assay and JC-1 assay were used to determine the loss of MMP induced by yuanhuatine treatment in MCF-7 cells (▶ Fig. 3 A–D). To examine whether collapse of mitochondrial membrane could induce ROS production, we next investigated the intracellular ROS levels using H₂DCF-DA staining. The results demonstrated that yuanhuatine significantly induced ROS generation (▶ Fig. 3 E, F). Meanwhile, mitochondrial superoxide accumulation was also measured using MitoSOX Red reagent. Treatment with yuanhuatine showed a significant increase of mitochondrial superoxide production as reflected by the increased of red fluorescence in MCF-7 cells (▶ Fig. 3 G, H).

The Bcl-2 protein family, including the anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax, is crucial for the regulation of mitochondrial apoptosis [17]. Yuanhuatine increased the Bax/Bcl-2 ratio, which was 3.5-fold (0.5μ M) than untreated cells (**> Fig. 31, J**). Taken together, these data demonstrated that yuanhuatine induces mitochondrial dysfunction and mitochondrial apoptosis in MCF-7 cells.

As shown in the above results, $ER\alpha$ -positive cells were more sensitive to yuanhuatine treatment compared to $ER\alpha$ -negative cells. To investigate whether $ER\alpha$ plays a key role in yuanhuatinetreated breast cancer cells, computational molecular docking



Fig. 1 Effect of yuanhuatine on cell viability in various cancer cell lines and normal MCF10A. A The chemical structure of yuanhuatine. B MCF-7, MM231 and MCF10A were treated with yuanhuatine at different concentrations $(0 - 100 \mu M)$ for 48 h, and the IC₅₀ values were quantified using the SRB assay. C MCF-7 cells were treated with yuanhuatine and tamoxifen at different concentrations $(0 - 100 \mu M)$ for 48 h, and cell viability was determined by SRB assay.

studies were performed to simulate the potential binding mode between yuanhuatine and ER α . Results from *in silico* studies reveal that yuanhuatine forms key hydrophobic interactions in the hydrophobic pocket with some crucial lipophilic residues (Phe 404, Leu 391, Leu 387, Ala 350, Leu 346, Met 421 and Leu 525) as seen in ligand estradiol binding (**> Fig. 4A, B**). This pocket could partly overlap with the ATP phosphate-binding region. Based on the above results, we deduce that yuanhuatine could bind to ER α .

To evaluate the expression of ER α in yuanhuatine-treated MCF-7 cells, we examined the expression of ER α protein using immunofluorescence staining. As shown in **>** Fig. 5A, B, compared with the control group, the fluorescence intensity of ER α per area is significantly decreased after yuanhuatine treatment, indicating that treatment with yuanhuatine reduces the level of ER α expression both in the nucleus and the cytosol. Numerous cases have shown that Akt/mTOR and MEK/ERK signaling molecules are important downstream molecules of ER α [18, 19]. We next used Western blot to test the protein expression of ER α and downstream molecules, which were regulated by yuanhuatine (**>** Fig. 5C, D). These data confirmed that yuanhuatine inhibited ER α signaling pathway in MCF-7 cells. To further elucidate the effect of ER α on yuanhuatine-induced cell death in MCF-7 cells, MPP, the specific ER α -antagonist was applied in the following experiments. The results from > Fig. 6 indicated that cell viability decreased significantly when the cells were co-incubated with yuanhuatine and MPP (5 μ M). MPP (5 μ M) alone showed no significant cytotoxicity. Annexin V-FITC/PI analysis and Western blots were performed to confirm yuanhuatine-induced apoptosis via ER α (> Fig. 7). As shown in > Fig. 8 compared with yuanhuatine-treated group, co-treatment with MPP resulted in a further reduced MMP and more increased of ROS levels. Yuanhuatine increased the Bax/Bcl-2 ratio, while co-treatment with MPP increased the ratio furthermore (> Fig. 9). The results indicate that ER α down-regulation contributes to mitochondrial dysfunction and hence apoptosis in yuanhuatine-treated MCF-7 cells.

Besides, we examined the expressions of downstream signaling molecules of ER α pathway. The levels of mTOR, p-Akt, p-ERK and ERK were further decreased after co-treatment with yuanhuatine and MPP (**Fig. 10**). These data confirmed that yuanhuatine inhibited ER α expression and further down-regulated the downstream molecules.

To further confirm the effect of yuanhuatine in $ER\alpha$ -positive breast cancer cells, MM231 cells were examined by flow cytome-



► Fig. 2 Yuanhuatine-induced apoptosis in MCF-7 cells. A Morphological changes of MCF-7 cells during the culture with DMSO or 0.25, 0.5, 1 µM of yuanhuatine for 48 h were observed with a phase contrast microscope (200×). Cell shrinkage and rounding of the cell shape were observed. Representative images of MCF-7 cells with 50 µm scale bars are shown. B MCF-7 cells were treated with DMSO or 0.25, 0.5, 1 µM of yuanhuatine for 48 h and stained with Hoechst 33 258. The morphological changes of nuclei were examined by fluorescence microscopy (200×). Representative images of MCF-7 cells are shown with white arrows indicating apoptotic cells. Scale bar; 50 µm C MCF-7 cells were treated with yuanhuatine for 48 h, stained with Annexin V-FITC in a buffer containing PI, and analyzed by flow cytometry. D, E The expressions of pro-caspase-7, cleaved caspase-7 and PARP were analysed by western blotting. F MCF-7 cells were pretreated with or without Z-VAD-fmk for 1 h, followed by the treatment with 0.5 µM yuanhuatine for another 48 h. Cell viability was determined by SRB assay. G Apoptosis was analyzed by flow cytometry. The results are expressed as means ± SD for each group. **p < 0.01; ***p < 0.001 versus control. ^{&®}p < 0.01; compared with yuanhuatine alone.</p>



Fig. 3 Yuanhuatine-triggered apoptosis is associated with mitochondrial dysfunction. **A**, **B** The MMP of MCF-7 cells treated with yuanhuatine (0, 0.25, 0.5, 1 μM) for 48 h, was obtained by staining with Rhodamine 123 followed by flow cytometric analysis. **C**, **D** The MMPs of MCF-7 cells were determined by JC-1 staining, followed by flow cytometric analysis. **E**, **F** ROS levels were determined by flow cytometry after staining with H₂DCF-DA. **G**, **H** Mitochondrial superoxide production was evaluated by MitoSOX staining with a fluorescence microscope (200×). **I**, **J** The levels of Bax and Bcl-2 were determined by western blotting. The results are expressed as means ± SD for each group. **p < 0.01; ***p < 0.001 versus control.



Fig. 4 Molecular docking studies. **A** The binding mode of ligand in the active site of ERα pocket. Hydrophobic surface of ligand towards ERα pocket. **B** The binding mode of yuanhuatine in the active site of ERα pocket. Hydrophobic surface of yuanhuatine towards ERα pocket. Yuanhuatine (yellow carbon) in contact with amino acids residues (cyan carbon) were presented in sticks. Hydrophobic interactions were highlighted in carnation dashed lines. Hydrophobic surface in receptor residues were indicated from blue (hydrophilic) to brown (hydrophobic).

try analysis. As expected, MM231 cells were resistant to yuanhuatine (Fig. 1S, Supporting Information), which may indicate that necessity of the change from upregulation to downregulation of ER α in yuanhuatine-induced apoptosis.

Discussion

ER α is a member of the steroid receptor superfamily that regulates processes such as growth and differentiation in various target cells [20]. Currently, the most popular classification of breast cancer is according to the ER α status in selecting appropriate chemotherapeutic drugs [21]. Clinically, selectively inhibition of ER α has long been considered a critical and effective strategy to prevent the development and progression in breast cancer [22]. Up to date, various natural materials have been reported to inhibit breast cancer cells growth by down-regulating ER α expression [23–26]. Therefore, it is of great importance to develop novel chemicals, which are more selective to ER α , but have fewer side effects for the treatment of breast cancer. Natural products were potential sources of chemotherapy for breast cancer [27]. Daphnane-type diterpenoids are known to be the main types of plant diterpenoid orthoesters with significant biological activity [28]. Yuanhuatine, a natural daphnane-type diterpenoid deriving from *Daphne genkwa*, exhibits significant inhibitory effect in 10 selected human cancer cell lines in our previous study [11]. However, detailed mechanism of anti-tumor effect of yuanhuatine in breast cancer cells has not been reported. In this study, our results demonstrate that yuanhuatine induces mitochondrial apoptosis by repression of ER α level.

Due to the potent anti-tumor effects of yuanhuatine, we further predicted the pharmacokinetic properties and drug-likeness properties of yuanhuatine using PreADMET web server (http:// preadmet.bmdrc.kr). From **Table 1S** (Supporting Information), we identified that yuanhuatine exhibited good predicted pharmacokinetic properties. In addition, Ames test suggested yuanhuatine showed non-toxicity. These results suggest that yuanhuatine has a therapeutic potential for the treatment of breast cancer.





In summary, our studies have elucidated the notable anti-tumor effect of yuanhuatine via down-regulation of ER α followed by mitochondrial dysfunction and mitochondrial apoptosis in ER α -positive breast cancer cell lines. Therefore, yuanhuatine may provide a promising candidate for the treatment of ER α -positive breast cancer.

Materials and Methods

Plant material

The flower buds of *D. genkwa* were collected from Mianyang, Sichuan province, PR China, in June 2010, and were identified by Professor J. C. Lu (Department of Natural Products Chemistry, Shenyang Pharmaceutical University, PR China). A voucher specimen (No. 20100701) has been deposited in the Herbarium of Shenyang Pharmaceutical University, Liaoning, PR China.



► Fig. 6 MPP (an ER α -antagonist) was applied with yuanhuatine to examine the cell viability of MCF-7 cells. The cells were pre-treated with MPP 1 h prior to the administration of yuanhuatine (0.5 μ M) that lasted for 48 h, and the cell viability was measured by SRB assay. **p < 0.01 versus control; ^{&&}p < 0.01; ^{&&&}p < 0.001 compared with yuanhuatine alone.

Chemicals and reagents

Yuanhuatine was dissolved in dimethylsulfoxide (DMSO) at a stock concentration of 50 mM. Sulforhodamine B (SRB), rhodamine 123, DAPI and ERα-specific antagonist MPP 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2- piperidinylethoxy)phenol]-1H-pyrazole-dihydro-chloride were purchased from Sigma-Chemical. Fetal bovine serum (FBS) was purchased from CLARK Bioscience. Dulbecco's modified Eagle's medium (DMEM), Phosphate buffered solution (PBS) and Antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin) were purchased from HyClone, Inc. caspase-7, Bax, Bcl-2, ER α , p-Akt, Akt, mTOR, MEK, p-ERK, ERK, β -actin and horseradish-peroxidase-conjugated secondary antibodies (goat anti-rabbit or goat anti-mouse) were purchased from Santa Cruz Biotechnology. Dichlorofluorescein diacetate (H₂DCF-DA), Hoechst 33258, JC-1, RIPA lysis buffer, BCA assay kit were purchased from Beyotime. Annexin V-FITC was purchased from Bimake. MitoSOX Red mitochondrial superoxide indicator was purchased from Invitrogen. PVDF membranes (0.2 µm) were purchased from Millipore.

Cell culture

MCF-7, MM231 and MCF10A cells were obtained from American Type Culture Collection. Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were incubated at







Fig. 8 ER α was involved in the regulation of mitochondrial function of yuanhuatine-treated MCF-7 cells. A, B The MMP was measured by flow cytometry with JC-1 staining. C Flow cytometry analysis was used to assess the intracellular accumulation of ROS. [&]p < 0.05 compared with yuanhuatine alone.

 $37 \,^{\circ}$ C with $5\% \,$ CO₂ in a humidified atmosphere. All experiments were performed on logarithmically growing cells.

SRB assay

Sulforhodamine B (SRB) assay was employed for measurement of *in vitro* growth inhibition and cytotoxicity. Breast cancer cells were seeded at a cell density of 5×10^3 cells/well in 96-well plates and incubated for 24 h to allow exponential growth, then treated with yuanhuatine. After 48 h of treatment, 50% trichloroacetic acid (TCA) was added to the culture medium, for 1 h at 4 °C in the dark, to fix the cells. The plates were then washed with tap water to remove TCA, air-dried and stained for 30 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. After incubation, the cultures were rinsed with 1% acetic acid to remove residual dye, the plates were air-dried and the bound dye was solubilized with 200 µL of 10 mM Tris buffer (pH 10.5). The absorbance of each well was determined at 540 nm using a microplate reader. Cell growth inhibitory ratio was calculated using the following equation:

Observation of morphological changes

Cells were seeded into 12-well plates (1×10^5 cells/well) and incubated for 24 h to allow exponential growth, then incubated in the presence or absence of yuanhuatine at the indicated concentrations for 48 h. The cellular morphology was observed by using a phase contrast microscope. Apoptotic nuclear morphology was assessed by fluorescent DNA-binding dye, Hoechst 33258. After incubation, the culture medium was removed. The cells were fixed for 15 min in 4% paraformaldehyde, and then incubated in Hoechst 33258 ($5 \mu g/mL$) at room temperature for 30 min. Finally, a fluorescence microscope was used to examine the chromatin condensation.

Cell apoptosis ratio detection

Cells were seeded into 6-well plates (2 × 10⁵ cells/well) and incubated for 24 h to allow exponential growth, treated with yuanhuatine for 48 h. After incubation, the cells were washed twice in PBS and resuspended in an Annexin V-FITC/PI staining solution according to the manufacturer's instruction. Apoptotic cells were immediately analyzed by flow cytometry. The samples are then analyzed with Flow Jo 7.6.5.



▶ Fig. 9 The expression levels of Bax and Bcl-2 were determined by Western blotting in yuanhuatine-treated cells with or without MPP. ^{&&&}p < 0.001 compared with yuanhuatine alone.





Western blot analysis

The total cellular samples were harvested and lysed in RIPA buffer [250 mM Tris-HCl (pH 6.8), 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β -mercaptoethanol, 50 mM sodium fluoride, and 5 mM sodium orthovanadate] and boiled for 10 min at 100 °C. Equal amount of protein (35 µg) were separated on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were blocked with 5% BSA and probed with a primary antibody (1:1000) followed by the corresponding secondary antibody (1:5000). Immunoreactive bands were visualized with a chemiluminescence kit followed by incubation with HRP-conjugated secondary antibodies. The density of protein bands were calculated by the ImageJ software.

Mitochondrial membrane potential (MMP) analysis

MMP was measured by flow cytometry analysis using Rhodamine 123. Cells (2×10^5 cells/well) were plated in 6-well plates and incubated for 24 h to allow exponential growth. The cells were treated with the yuanhuatine for 48 h. The treated cells were collected and analyzed immediately for Rhodamine 123 fluorescence intensity by flow cytometer.

JC-1 assay kit was used to evaluate MMP as well. Breast cancer cells collected were supplemented with 500 μ L of JC-1 dye staining solution, and then incubated in the dark at 37 °C for 20 min. After incubation, the cells were centrifuged at 600 g for 5 min and washed twice with incubation buffer. The fluorescence levels were then determined by flow cytometry after the cells were resuspended in 500 μ L of the incubation buffer. The samples are then analyzed with Flow Jo 7.6.5.

Measurement of intracellular ROS levels

The intracellular reactive oxygen species (ROS) levels were evaluated using the ROS-specific fluorescent dye H₂DCF-DA (10 μ mol/L). After indicated treatments, cells were washed with PBS for three times and stained with H₂DCF-DA, incubated at 37 °C for 30 min in the dark. Furthermore, cells were harvested and the levels of ROS were quantified by measuring the intracellular florescent intensities by flow cytometry. Data were then analyzed with Flow Jo.7.6.1.

Mitochondrial superoxide

To analyze mitochondrial superoxide levels, cells were incubated with MitoSOX (5 μ M) for 10 minutes at 37 °C. PBS was used to wash the cells and superoxide levels were determined using a fluorescence microscope at 530/590 nm.

Immunofluorescence staining

MCF-7 cells were washed with PBS, fixed in 4% paraformaldehyde for 20 min at room temperature, and permeabilized in 0.1% Triton X-100 for 10 min. After blocking with 5% bovine serum albumin (BSA) for 20 min, the samples were incubated with anti-ER α antibody (1:500) overnight at 4°C, followed by reaction with a FITClabelled secondary antibody (1:1000) at room temperature for 1 h. After being washed with PBS, the cells were incubated with 4, 6-diamidino-2-phenylindole dihydrochloride (DAPI) to stain the nucleus and observed under a fluorescence microscope.

Molecular docking

The X-ray crystal structure of the ER α (PDB: 5 U2D) and a resolution of 1.86Å was obtained from the RCSB protein data bank (PDB) (http://www.wwpdb.org). The protein was optimized by the Sybyl-X (version 2.0, TRIPOS Inc.) software to add all hydrogen atoms and remove water molecules. The structural relation was carried out for the docking calculations using the AUTODOCK 4.2 SUITE of program. The visualization of resulting complex geometry was performed on Discovery Studio 4.5 program.

Statistical analysis

All results were calculated as means \pm SD for at least three independent experiments. Experimental data were analyzed by oneway or two-way ANOVAs using GraphPad Prism version 6.0. Statistical significance was considered at *P* < 0.05

Supporting Information

Additional information about the induction of cells apoptosis by yuanhuatine treatment of MM231 cells and the pharmacokinetic properties of yuanhuatine predicted by PreADMET web server are available in Supporting Information section.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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