Original Paper

Antiestrogenic Activity of the Xi-Huang Formula for Breast Cancer by Targeting the Estrogen Receptor α

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Key Words

XH formula • ER α • HSP90 • Breast cancer • Commiphora Myrrha • Olibanum

Abstract

Background/Aims: The Xi-Huang (XH) formula has been used for breast cancer treatment in traditional Chinese medicine (TCM) since 1740. In this study, we show that, XH extract could suppress the growth of breast cancer cells *in vitro* and *in vivo*, and that it preferentially inhibits cell growth of estrogen receptor positive (ER+) breast cancer cells. Presently, little is known about the potential mechanism of XH and our studies aim to elucidate its mechanism in breast cancer treatment. **Methods:** Network-based systems biology and molecular docking analyses were performed to predict explicit targets of XH and active ingredients in XH. The effects of XH on cell viability, cell cycle, apoptosis in different breast cancer cell lines were analyzed *in vitro*. A model of transplanted tumors on nude mice was used to study the anticancer effect *in vivo*. Various techniques, including western blotting, reverse transcription-polymerase chain reaction (RT-PCR), immunofluorescence, co-immunoprecipitation and immunohistochemical were utilized to assess the expression of targets of XH *in vitro* and *in vivo*. RNA sequencing (RNA-seq) was performed to study the gene targets of XH. Furthermore, we analyzed of protein-ligand binding reactions by isothermal titration calorimetry (ITC). **Results:** Using network-based systems biology and molecular docking analyses, we predicted that the major targets of XH were ER α and HSP90. Moreover, we found that, XH mediated its anti-cancer effects by promoting the disassociation of ER α and HSP90, resulting in the degradation of ER α and blockade of transport of ER α to the nucleus. XH also caused the dissociation of ER α and other oncoproteins via binding to HSP90. Some of the active ingredients in XH share a common cyclopentane hydrogen skeleton and were predicted to target ER α based on the

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structural similarity. **Conclusions:** XH, which has been used since 1740, has antiestrogenic effects in breast cancer via the targeting of ER α .

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Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females worldwide [1]. Over 70% of breast cancer patients express estrogen receptor (ER) and are thus candidates for hormonal therapy [2]. However, till 1977, the ER-targeting agent tamoxifen was approved for treatment of post-menopausal women with advanced breast cancer [3]. Until 2002, the pure estrogen antagonist Fulvestrant was approved for breast cancer [4]. Fulvestrant competitively inhibits the binding of estradiol to the ER, prevents its dimerization, and impairs energy-dependent nucleo-cytoplasmic shuttling. More importantly, the binding of fulvestrant to ER α initiates the dissociation of HSP90 from ER α , followed by receptor degradation via the ubiquitin-proteasome pathway [5-7]. HSP90 is a molecular chaperone protein that plays an essential role in folding and stabilizing cellular proteins [8]. Its ability to stabilize ER α , Akt, Raf, EGFR and HER2 suggests a crucial role for HSP90 in maintaining the survival of breast cancer cells [9, 10]. Consequently, targeting ER α and its partner HSP90 has become an important strategy in the treatment of breast cancer.

The XH formula has been used for breast cancer treatment in traditional Chinese medicine (TCM) since 1740, as recorded in the *Wai Ke Quan Sheng Ji* [11]. Subsequently, the wide application of the XH formula in breast cancer has been recorded in *Xu Mingyi Lei'an* in 1770 [12], *Wai Ke Zheng Zhi Quan Shu* in 1831 [13] and *Yan Fang Xin Bian* in 1878 [14]. The formula contains four ingredients: *Olibanum* (Oli), *Commiphora Myrrh* (CM), *Moschus* (M) and *Calculus Bovis* (BC). Modern pharmacological studies have established the anti-breast cancer effects of guggulsterone (GS), one of the major ingredients of CM, and boswellic acid,

Fig. 1. Process overview

one of the major ingredients of Oli [15-18]. However, the complicated mode of XH in breast cancer has not been researched.

In this study, we applied network-based systems biology and molecular docking analyses to predict and verify the explicit targets and active ingredients in the XH formula. The predicted results were evaluated by both *in vitro* and *in vivo* experiments (Fig. 1). We found that XH, which has been used since 1740, has antiestrogenic effects in breast cancer via targeting ER α .

Materials and Methods

Drugs and Reagents

The crude extract of XH and its components was provided by Jiangsu Wanbang Biopharmaceuticals (Jiangsu, China). Guggulsterone was obtained from Cayman (USA, NO. 39025-24-6). Acetyl-alpha-boswellic acid was purchased from Yuanye Biotechnology Development Company (Shanghai, China, NO. C04N7G24096). Boswellic acid (NO. 0002555-003) and acetyl-beta-boswellic acid (No. 0002565-A001) were obtained from ChromaDex (USA). Fulvestrant (Lot.14), tamoxifen (Lot.04) and 17-AAG (Lot.02) were purchased from Selleck (USA). ER α (ab16660), HSP90 (ab79849), Akt (ab8805), EGFR (ab52894), Raf (ab200653) and β -actin (ab8227) were bought from Abcam (USA). Erk (4695) and HER2 (2842S) were obtained from Cell Signaling Technology (USA). The proteasome inhibitor MG132 was obtained from Calbiochem (San Diego, CA). The ER α protein (Ser178- Met438, which includes the ligand binding domain of estrogen receptor) was purchased from Cloud-Clone CORP (China).

Cell Culture

MCF-7, T47D and SKBr3 cells were cultured in DMEM (Invitrogen, Germany) with 10% fetal calf serum at 37°C in a humidified atmosphere of 95% air and 5% CO₂. MDA-MB-231 cells were cultured in Leibovitz's L-15 medium with 10% fetal calf serum (FCS) without CO₂. MCF-7, T47D, SKBr3 and MDA-MB-231 cells were from Peking Union Medical University. To reduce hormone interference during certain experiments, the cells were maintained in phenol red free medium containing 5% charcoal-stripped serum for 4 days prior to use.

Cell Proliferation Assay

Cell viability and proliferation were examined by CCK-8 assays (Sigma) and trypan Blue staining. SKBr3, MCF-7, T47D and MDA-MB-231 cells were seeded onto 96-well plates (2000-3000 cells/well). WST-8 was added into each well. After incubation, the absorbance values were determined by microplate luminometer (Bio-Rad, USA) at 450 nm. Exponentially growing breast cancer cells ($1-2 \times 10^4$) were plated in 24-well plates. Following treatment, cells were counted using 0.4% trypan blue to verify viability.

Cell cycle and Cell apoptosis Analysis

Cells (2×10^5) were seeded onto a 6-well plate. After treatment, the cells were harvested and stained with Annexin V-FITC and propidium iodide (BD, New York, USA). Then, cell apoptosis was analyzed by FACSCalibur flow cytometry (BD, New York, USA). To determine the cell cycle distribution, cells were harvested and stained with 50 mg/mL PI.

Candidate BC-related Targets

Candidate BC targets were obtained from the Therapeutic Target Database (http://bidd.nus.edu.sg/group/cjttd/ttd_home.asp) [19] and widely cited studies [20-23].

Herb Formulation Ingredient Collection and Target Fishing

As in our previous research [24], the chemical ingredients were obtained from the Traditional Chinese Medicine Systems Pharmacology (TCMSP) Database (<http://lsp.nwsuaf.edu.cn/tcmsp.php>) [25], and ChemMapper (<http://lilab.ecust.edu.cn/chemmapper/>) [26]. They were subsequently screened, according to drug-likeness (DL) values, considering both pharmaco-dynamics and pharmaco-kinetic properties.

Network Construction and Analysis

The predicted targets were matched with hub breast cancer-related proteins. Ingredients with total predicted scores > 0.5 and single predicted score > 0.01 were further analyzed by Cytoscape software [27] and by the CentiScaPe plugin [28] to calculate topological parameters.

Docking Exercises of Ingredients Binding to ER α and HSP90

The crystal structure of ER α was obtained from the Protein Data Bank (PDB ID: 1A52) [29]. The crystal structure of HSP90 was also obtained from the Protein Data Bank (PDB ID:3EKO) [30]. The docking exercise was conducted through systemsDock (<http://systemsdock.unit.oist.jp/iddp/home/index>) [31].

Western Blot Analysis

Cell lysate proteins were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. Each membrane was incubated with monoclonal antibodies against ER α (1:800), HSP90 (1:1000), Raf (1:2000), Akt (1:2000), Erk (1:2000), EGFR (1:2000), ErbB3 (1:2000), HER2 (1:2000) and β -actin (1:2000), followed by incubation with peroxidase-conjugated secondary antibodies and chemiluminescence detection.

Immunofluorescent Assay

2×10^4 cells were fixed on cover glasses and incubated with rabbit anti-ER α antibody (1:250) overnight and then with FITC-conjugated anti-rabbit IgG antibody. Glass was covered with mounting medium containing DAPI (4',6-diamidino-2-phenylindole, HelixGen Anti-fade Fluorescence Mounting Medium, China). Images were acquired using a confocal microscope (Olympus).

Immunohistochemical Analysis

5 mm thin sections of tumor samples were deparaffinized and stained with primary antibodies against ER α (dilution 1: 250) and PCNA (dilution 1: 250) and then with the secondary antibody. Next, were stained with hematoxylin.

TUNEL Assay

Apoptosis in tumor tissue was detected with the In Situ Cell Death Detection Kit (Roche Diagnostic-Mannheim, Germany). Briefly, after fixing and permeabilization, the tissue was incubated with the TUNEL reaction mixture. And then was covered with mounting medium containing DAPI. Representative images were acquired using a confocal microscope (Olympus).

Co-immunoprecipitation

After the different treatments, cells were collected and resuspended in a lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 1.0% IGEPAL® CA-630, 0.5% Triton X-100 and protease inhibitors. Cell lysates were incubated with ER α antibody or IgG and then incubated with protein A/G-agarose beads. The agarose beads were then centrifuged, washed, and then resuspended in SDS-PAGE loading buffer for western blot.

RNA Extraction, cDNA Library Construction and Illumina Sequencing

Total RNA was extracted using TRIzol reagent (Invitrogen). RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN). The library fragments were purified with the AMPure XP system (Beckman Coulter). The cDNA was then amplified by bridge amplification to generate clonal DNA clusters, and the amplified PCR products were purified using AMPure XP beads (Beckman Coulter). After cluster generation, the library preparations were sequenced on an Illumina HiSeq 2000/2500 platform, and 100 bp/50 bp single-end reads were generated.

Real-time Reverse Transcription-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). cDNA was synthesized from 2 μ g of total RNA using reverse transcriptase. Real-time PCR was carried out with 2 μ l of cDNA and SYBR Green Master Mix (Qiagen). The forward and reverse primers used for ESR1, HSP90 and GAPDH were as follows: ESR1, 5'-GAAGTGAAGAACGTGGTG-3' and 5'-ATGCGATGAAGTAGAGCC-3'; HSP90, 5'-CCCTTCTATTTGTCCC-3' and

5'-ATGCGATGAAGTAGAGCC-3'; GAPDH, 5'-CCACTCCTCCACCTTTG-3' and 5'-CACCACCCTGTTGCTGT-3'. The thermal conditions were as follows: 3 min at 95°C, followed by 39 cycles of 95°C for 5 s, 56°C for 10 s, 72°C for 25 s, 65°C for 5 s, and 95°C for 50 s.

Tumor Xenograft Assay

Female BALB/c nude mice aged 4 weeks (Vital River Experimental Animal Technical Company, Beijing) were injected subcutaneously with T47D and SKBr3 breast cancer cells (0.1 mL, 1×10^7 cells). After 10 days, necrotic and non-cancerous tumors were removed and divided into approximately 8-mm³ fragments. A fragment of tumor was subcutaneously implanted using an implanting needle (Natsume, Tokyo, Japan). The mice with T47D xenografts were allocated into six groups: 1) normal saline (n = 10); 2) XH 150 mg/kg/d (n = 8); 3) CM 75 mg/kg/d (n = 8); 4) Oli 75 mg/kg/d (n = 8); 5) CM 75 mg/kg/d + Oli 75 mg/kg/d (n = 8); 6) fulvestrant 5mg/weekly (n = 8). The mice with SKBr3 xenografts were allocated into five groups: 1) normal saline (n = 8); 2) XH 150 mg/kg/d (n = 8); 3) CM 75 mg/kg/d (n = 8); 4) Oli 75 mg/kg/d (n = 8); 5) CM 75 mg/kg/d + Oli 75 mg/kg/d (n = 8). The administration of XH, CM, Oli, and CM+Oli began on the next day of inoculation via intragastric injection. Fulvestrant was used once weekly by intraperitoneal injection. Tumors were measured individually every three days. The tumor volume (mm³) was calculated as $V = \text{length} \times \text{width}^2 / 2$. On the 17th day, the animals were weighed and sacrificed. The implanted tumors were excised and weighed. All experiments were approved by the Tianjin Cancer Institutional Animal Ethics Committee (#2016088).

Isothermal Calorimetry(ITC)

ITC experiments were performed on a MicroCal VP-ITC MicroCalorimeter (MicroCal, Northampton, MA) calibrated as per the recommended protocol. To prepare the ligand solution, 500 µmol of estradiol and guggulsterone was dissolved in 1% DMSO and 99% PBS. A matched quantity of DMSO and PBS was added to the protein sample. All solutions were degassed for 10–15 min at 20°C before loading the samples in the ITC cell and syringe. All titrations were carried out at 25°C with a stirring speed of 351 rpm and 180 s spacing between successive 10 µl injection. To correct for the heat of dilution, control experiments were performed by making identical injections of the titrand solution into a cell containing only buffer with an equal concentration of DMSO. These control experimental values were subtracted from their respective titration data before data analysis.

Statistical Analysis

Graphs were generated using Excel, PowerPoint software program, GraphPad Prism 5 and ggplot2. Statistical significance was determined using the Statistical Package for the Social Sciences (SPSS) 12.0. All experimental data were analyzed by one-way analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons, and there was no significant difference in the variance homogeneity test ($P > 0.05$).

Results

Higher Levels of Growth Inhibitory Effects Were Observed in ER+ Breast Cancer Cells

The effects of XH and its components on the viability of the ER+ breast cancer cell lines MCF-7 and T47D as well as ER- cell lines SKBr3 and MDA-MB-231 were evaluated. As shown in Fig. 2A, while each component of XH individually induced a certain degree of growth inhibition in all of these breast cancer cells lines, the strongest effect was observed after XH treatment. In XH formula, the ratio of the four herbs (CM: Oli: M: BC) was 30: 30: 4.5: 1. But the half maximal inhibitory concentrations of BC and M were higher than those of CM and Oli. Therefore, M and BC may not be essential for the direct inhibitory effects of XH. Moreover, the inhibitory effect of XH on SKBr3 and MDA-MB-231 cells was lower than that on MCF-7 and T47D cells. At a concentration of 50 µg/ml, XH could half-inhibit the proliferation of MCF-7 and T47D cells, while in SKBr3 and MDA-MB-231, that was close to 150 µg/ml. These results suggested that XH is more potent in reducing the proliferation of ER+ cells than that of ER- cells.

Fig. 2. Higher levels of growth inhibitory effects in ER+ breast cancer cells. The growth inhibitory effects of XH and its components were measured in four breast cancer cell lines at different concentrations (A). XH (50 µg/ml), CM (100 µg/ml), and Oli (150 µg/ml) induced a certain degree of growth inhibition in ER+ cells through increasing the cell apoptosis. And XH (100 µg/ml), CM (150 µg/ml), and Oli (200 µg/ml) also induced the apoptosis of ER- cell lines (B). XH (50 µg/ml), CM (100 µg/ml), and Oli (150 µg/ml) in ER+ cells and XH (100 µg/ml), CM (150 µg/ml), and Oli (200 µg/ml) in ER- cell lines had effects on cell cycle distribution (C).

Effects of XH and Its Components on the Biological Characteristics of ER+ Breast Cancer Cells

Consistent with the results of the cell viability assays, there was a significant increase in apoptosis treated with XH (50 µg/ml), CM (100 µg/ml), and Oli (150 µg/ml) for 24h in ER+ breast cancer cell lines. The apoptosis rates were 52.0%, 46.3% and 36.9% respectively in the MCF-7 cells and 73.0%, 53.6% and 18.4%, respectively, in the T47D cells (Fig. 2B). After 24 h, XH (100 µg/ml), CM (150 µg/ml), and Oli (200 µg/ml) also induced the apoptosis of ER- cell lines. The apoptosis rates were 45.2%, 26.1% and 17.6% respectively in the SKBr3 cells and 34.4%, 29.1% and 12.5%, respectively, in the MDA-MB-231 cells. XH, CM, and Oli also interfered with the cell cycle of these breast cancer cell lines, which was reflected by a distinct increase in the percentage of cells in the G₀/G₁ phase and a significant reduction in those in the S and G₂/M phases (Fig. 2C). The results of cell apoptosis and cell cycle experiments again indicated that the effects of XH and the components CM and Oli on MCF-7 and T47D were more obvious than that in the SKBr3 and MDA-MB-231 cells.

Effects of XH, CM and Oli on Xenograft Tumor Models

XH and its components CM and Oli alone showed potent therapeutic efficacy in both T47D and SKBr3 xenografts (Fig. 3A, B). For T47D xenografts, compared with the controls, XH treatment suppressed the growth of transplanted tumors by 72.50% ($P < 0.001$). The suppression rates for CM (75 mg/kg/d), Oli (75 mg/kg/d) and CM+Oli were 51.93%, 48.46% and 68.03%, respectively ($P < 0.001$ for all). XH remarkably and effectively suppressed tumor growth compared to either component alone, with a reduction in tumor weight of

Fig. 3. Effects of XH, CM and Oli on Xenograft Tumor Models. XH and its components CM and Oli alone showed potent therapeutic efficacy in both T47D (A) and SKBr3 xenografts (B). The levels of PCNA-positive cells decreased after treatment with XH or its components (C) and the distribution of the TUNEL-positive nuclei was higher after the treatment of XH or its components compared with the control group (D). No drug-related adverse effects were observed (E).

42.87% ($P = 0.010$, v.s. CM) and by 46.87% ($P = 0.003$, v.s. Oli). The combination of CM+Oli also suppressed tumor growth, with a tumor weight reduction of 33.49% ($P = 0.041$, v.s. CM) and by 38.14% ($P = 0.014$, v.s. Oli). Compared to the combination of CM+Oli, XH had some advantage in tumor suppression, but there was no statistical significance ($P > 0.05$). The same treatments of XH and its components CM and Oli alone showed advantage in T47D xenografts. For SKBr3 xenografts, XH treatment suppressed the growth of transplanted tumor by 56.05% compared with the controls. The suppression rates of CM (75 mg/kg/d), Oli (75 mg/kg/d) and CM+Oli were 26.90%, 33.54% and 55.68%, respectively.

Proliferating cell nuclear antigen (PCNA) expression analysis was performed to evaluate cell proliferation by immunohistochemistry. The levels of PCNA-positive cells decreased after treatment with XH or its components (Fig. 3C). Apoptosis levels *in situ* were analyzed by TUNEL assay, and the distribution of the TUNEL-positive nuclei was higher after the treatment of XH or its components compared to that in the control group (Fig. 3D).

XH treatment did not lead to toxicity in mice. As shown in Fig. 3E, the organ indices were in generally similar between the control group and each of the treatment groups. XH treatment also did not cause weight loss in animals ($P > 0.05$).

Active Ingredients in XH and Their Targets for Breast Cancer Treatment

The Therapeutic Target Database (TTD) is a database that provides information about known and explored therapeutic targets. We searched the TTD database and obtained 84 breast cancer-related target candidates. The remaining 36 target candidates were obtained from widely cited reasearches. The ingredients and possible targets for XH formula were predicted using TCMSP and TCMID databases. The predicted targets were matched with hub breast cancer-related proteins. Ingredients with total predicted scores > 0.5 and single predicted score > 0.01 were selected to construct an ingredient-target (ci-ct) network. As

Table 1. The major ingredients in XH formula and their docking scores

Herbal Name	Ingredients	Predicted Scores	Major Targets	Molecular Docking Scores	
				ERα PDBID:1A52	HSP90 PDBID:3EKO
Commiphora Myrrha					
	20α-hydroxy-4-pregnen-3-one (20αHP)	1.534	ERα;AKT;HSD17B11	7.86	
	Guggulsterone	0.942	ERα;HSD17B11	7.65	
	Guggulsterol V	0.938	ERα; HSP90;Akt;CDK6;Src	8.40	8.22
	Guggulsterone M	0.850	ERα;Akt;Src	7.90	
	Guggulsterol-I	0.824	ERα; HSP90;Akt	8.34	8.10
	Native ligand (estrogen)		ERα	7.73	
Olibanum					
	Olibanumol I	0.933	HSP90;PTPN		8.30
	Acetyl-β-boswellic acid	0.621	HSP90;CDK4;DHFR;TOPII;VEGF		8.29
	Acetyl-α-boswellic acid	0.597	HSP90;CDK4;DHFR;TOPII;VEGF		8.33
	β-boswellic acid	0.544	HSP90;CDK4;DHFR;TOPII;VEGF		8.33
	Native ligand		HSP90		4.96

Fig. 4. (A) The ingredient-target networks of predicted ingredients. The diamond nodes represent ingredients; the circular nodes represent targets; and the colors of the nodes are illustrated from red to yellow in descending order of degree values. (B) predicted targets were listed according to the scores from high to low.

depicted in Fig. 4A, CM and Oli had more ingredients and putative targets for breast cancer treatment and were the major components of XH. The predicted targets were listed in Fig. 4B according to their scores. Among them, ER α and HSP90 were the major putative targets in XH formula, with the highest scores. The most relevant ingredients for breast cancer treatment were listed in Table 1 according to their predicted scores. Interestingly, most of the active ingredients in CM were predicted to target ER α due to the structural similarity, which were similar to fulvestrant. The major active ingredients of Oli were pentacyclic triterpenes (Fig. 5A).

Docking Exercises of Binding to ER α and HSP90

Computational docking exercises were conducted to mimic the characteristics of ingredient-target binding. The crystal structure of ER α was obtained from the Protein Data Bank (PDB ID: 1A52) [29]. The docking exercise was conducted using systemsDock (<http://systemsdock.unit.oist.jp/iddp/home/index>) [30]. The results indicated that the predicted ingredients in CM formed more interactions with ER α than estrogen with similar or higher docking scores compared to estrogen (Fig. 5B). Moreover, the docking scores for G-V and G-I binding to ER α were also higher than those of fulvestrant.

The crystal structure of HSP90 was obtained from the Protein Data Bank (PDB ID: 3EKO) [31]. As shown in Table 1 and Fig. 4D, the binding scores of acetyl- β -boswellic acid (A- β -BA), acetyl- α -boswellic acid (A- α -BA) and β -boswellic acid (β -BA), which are the major ingredients of Oli, were 8.29, 8.33, and 8.33, respectively. The binding scores of G-V and G-I in CM were 8.22 and 8.10, respectively. These docking scores were higher than that of the natural ligand binding to HSP90 (4.96).

XH, CM and Oli Caused Reduction of ER α and Reduced Nuclear ER α Protein Levels in vitro and in vivo by Direct Interaction

As ER α was one of the most important targets for the XH formula with the highest predicted score, the levels of ER α *in vitro* and *in vivo* after XH, CM and Oli treatment were determined using western blot analysis, immunofluorescence staining and immunohistochemical staining.

Western blot results showed that the levels of ER α in MCF-7 and T47D cells were clearly reduced after treated with XH (50 μ g/ml), CM (100 μ g/ml), Oli (150 μ g/ml) for 48 h. The down-regulatory effects of XH could be prevented by pretreating the cells with a 10 μ M concentration of the proteasome inhibitor, MG132 (Fig. 6A). These results suggested that XH may promote ER α degradation via a proteasome-dependent pathway. Immunohistochemical analysis also demonstrated the reduction of ER α expression in tumor xenografts after XH and its components treatment (Fig. 6B).

The interaction of ER α and XH was also investigated using isothermal titration calorimetry (ITC). GS was one of the major ingredients of XH, with a 7.65 docking score for binding to ER α (7.73 for estradiol binding to ER α). Therefore, analysis of the thermodynamic properties of ER α and XH was performed using the standard ingredient GS with a purity greater than 95% to decrease the interference of impurities. The interaction of ER α and estradiol (E $_2$) was also tested with the same ER α protein.

As shown in Fig. 6C (left), the energetics of estradiol compound binding to ER α was measured using ITC. The binding reaction was exothermic and exhibited a slope of the ITC curve indicating a binding reaction. The values of K $_a$, Δ H and Δ S were 652 ± 100 /M, -1770 ± 16.23 cal/mol, and 7.17 cal/mol/deg, respectively. The binding curve of GS and ER α was similar to that of estradiol and ER α . A downward position of the ITC titration peaks (Fig. 6C, right, top panel) and the resultant negative integrated heats (Fig. 6C, right, bottom panel) demonstrated that the association between ER α ligand binding domain (LBD) and GS, is an enthalpy-driven process. The values of K $_a$, Δ H and Δ S were 348 ± 24.4 /M, -2037 ± 24.4 cal/mol, and 5.06 cal/mol/deg, respectively.

Immunofluorescence staining showed that treatment with XH and CM could reduce the level of ER α expression, especially in the cell nucleus. Similar changes were observed after

Fig. 5. Docking Exercises of Binding to ER α and HSP90. (A) The molecular structures of ingredients in CM were similar to that of fulvestrant, and the molecular structures of ingredients in Oli were pentacyclic triterpenes. (B) Docking exercises of these ingredients binding to ER α and HSP90 were shown. All these ingredients docked similarly to or better than the native ligands.

treatment with fulvestrant. The treatment with Oli could decrease the ER α level in the whole cell, but obvious differences were not observed between the levels in the nucleus and the cytosol (Fig. 6D).

Alterations in mRNA expression in MCF-7 and T47D Cells After Treatment with XH

The effects of XH on the mRNA levels of ESR1 and HSP90 were explored using RT-PCR in both MCF-7 and T47D cells, as both ER α and HSP90 were the major predicted targets of XH (Fig. 5E). We found that XH treatment had no influence on the mRNA levels of ESR1 and HSP90, which showed that the decreased expression of ER α was a result of increased protein degradation but not the decreased transcription.

RNA sequencing (RNA-seq) was also employed to study mRNAs alterations in MCF-7 after treatment with XH. 381 mRNAs were significantly differentially expressed after XH

Fig. 6. XH, CM and Oli caused reduction of ERα and reduced nuclear ERα protein levels *in vitro* and *in vivo*. The levels of ERα in MCF-7 and T47D cells were clearly reduced after treated with XH, CM, Oli. The down-regulatory effects of XH could be prevented by pre-treating the cells with the proteasome inhibitor, MG132(A). Immunohistochemical analysis also demonstrated the reduction of ERα expression in tumor xenografts after XH and its components treatment (B). The direct interaction of major ingredient of XH and ERα was tested using ITC assay (C). Immunofluorescence staining presented that the treatment of XH disrupted the nuclear localisation of ERα (D). XH treatment had no influence on the mRNA levels of ERα and HSP90 in both MCF-7 and T47D cells (E). After XH treatment, mRNAs involved in cancer-related biological processes and pathways, such as cell apoptosis, cell cycle and the ErbB signaling pathway, the MAPK signaling pathway and the mTOR signaling pathway were down-regulated. The up-regulated genes were involved in cellular hormone metabolic process (F).

treatment, among which 334 mRNAs were downregulated and 47 were upregulated. The down-regulated genes were found to be involved in cancer-related biological processes and pathways, such as cell apoptosis, the cell cycle and the ErbB signaling pathway, the MAPK signaling pathway and the mTOR signaling pathway. The up-regulated genes were involved in cellular hormone metabolic processes (Fig. 6F).

XH Promotes the Dissociation of ER α and HSP90 Prior to ER α Degradation

The role of HSP90 in XH-triggered ER α degradation was examined, as inhibition of HSP90 function has been reported to cause degradation of ER α via a proteasome-dependent pathway [32]. We confirmed that HSP90 was associated with ER α in MCF-7 and T47D cells by co-immunoprecipitation. To prevent the degradation of ER α before the dissociation of ER α and HSP90, cells were pretreated with a 10 μ M concentration of proteasome inhibitor MG132. After treating T47D cells with XH (50 μ g/ml), CM (100 μ g/ml), or Oli (150 μ g/ml) for 48h, ER α was pulled down by the anti-HSP90 antibody, and the level of HSP90-associated ER α was significantly reduced (Fig. 7A). The total levels of HSP90 in MCF-7 and T47D cells were also measured after treating with XH and its components; as shown in Fig. 7B, the protein level of HSP90 did not change. Taken together, these results suggest that XH may directly target ER α for degradation without degrading HSP90, indicating that XH promotes the dissociation of ER α and HSP90 prior to ER α degradation.

Several sensitive HSP90 clients other than ER α have been implicated in the pathogenesis of breast cancer except ER α , including oncogenic signaling cascades [33-35]. Therefore, the expression of other HSP90 clients in breast cancer cells were measured using western blot. We found that the XH formula and its components could downregulate the expression of client proteins. As shown in Fig. 7C, downregulation of the HSP90 clients Raf, Akt and Erk was triggered by XH and its components alone in ER $^{+}$ cell lines MCF-7 and T47D. A significant downregulation of HER2, EGFR, Raf, Akt, and Erk in SKBr3 cells and ErbB3, EGFR, Raf, Akt, and Erk in MDA-MB-231 cells was also triggered by XH and its components (Fig. 7D). These results demonstrated that XH may act directly on HSP90; however, this topic requires additional investigation.

Fig. 7. XH promoted the dissociation of ER α and HSP90 before ER α degradation. The level of ER α was pulled down by the anti-HSP90 antibody and the level of HSP90-associated ER α was significantly reduced (A) but the protein level of HSP90 did not change (B). More importantly, significant degradation of HSP90 clients Raf, Akt, Erk in T47D and MCF-7 cells, was triggered by XH formula and its components alone (C, D).

Therapeutic Efficacy of the Predicted Ingredients in XH in Breast Cancer Cell Lines

After treated with different concentrations for 48 h to 72 h, these ingredients exhibited varying concentration-dependent inhibitory effects in different breast cancer cell lines. GS and 20 α HP, the ingredients that only bind to ER α , had obvious inhibitory effects on ER+ cell lines, but no effects on the SKBr3 and MDA-MB-231 cells. Both ER α inhibitors, fulvestrant and tamoxifen, also only inhibited the proliferation of ER+ cell lines (Fig. 8A). The active ingredients of Oli displayed no obvious difference among the ER+ cell lines and the others. The HSP90 inhibitor tanespimycin (17-AAG) was also potently cytotoxic to all the four cell lines, with IC50 values in a low nanomolar range (Fig. 8B).

Discussion

In the present study, we demonstrated that the XH formula and the components of CM and Oli can significantly suppress the growth of breast cancer cells via promoting cell

Fig. 8. Therapeutic efficacy of predicted ingredients of XH formula in breast cancer cell lines. GS and 20 α HP had no inhibitory effects in SKBr3 and MDA-MB-231 cell lines, as the two ingredients only bound to ER α . Both ER α inhibitors, fulvestrant and tamoxifen, also only inhibited the proliferation of ER+ cell lines (B). The active ingredients of Oli displayed no obvious difference among the ER+ cell lines and the others. The HSP90 inhibitor tanespimycin (17-AAG) was also potently cytotoxic to all the four cell lines, with IC50 values in a low nanomolar range.

apoptosis and affecting the cell cycle distribution *in vitro*. The inhibitory actions of XH were more obvious in ER+ breast cancer cell lines than in HER2+ cells and triple negative breast cancer cells (Fig. 2). The anti-breast cancer effects were also replicated in nude mouse transplanted tumor models (Fig. 3). We then applied a bioinformatics approach to detect the potential pharmacology of XH. Based on the network pharmacology analysis, we found that ER α and HSP90 were the major putative targets of XH formula, with the highest predicted scores (Fig. 4). Furthermore, the molecular structures of several highly effective ingredients in XH were similar to those of fulvestrant and showed high probabilities of binding to ER α in docking exercises. Previous studies have reported that celastrol, a kind of pentacyclic triterpene, could disrupt the HSP90-Cdc37 complex and result in the degradation of HSP90 client proteins [36, 37]. In our study, we found that, some major effective ingredients were also pentacyclic triterpenes with a remarkably high affinity for HSP90, as the docking scores of binding to HSP90 were higher than those of natural ligand (Fig. 5).

The predicted results were also further supported by examining the expression of ER α *in vitro* and *in vivo* following XH treatment. In the ER+ cell lines, we found that XH not only reduced the transport of ER α to the nucleus but also downregulated the expression of ER α *in vitro* and *in vivo* by direct interaction (Fig. 6A, B, C, D). The down-regulatory effects of XH could be prevented by pretreating the cells with the proteasome inhibitor, MG132. Moreover, XH treatment had no influence on the mRNA levels of ER α (Fig. 6E). Taken together, these results showed that the decreased expression of ER α was a result of increased protein degradation but not the decreased transcription. The level of ER α associated with HSP90 was significantly reduced after XH treatment, but the levels of HSP90 protein and mRNA were not altered (Fig. 6E, Fig. 7A, B). These results suggested that XH promoted the dissociation of ER α and HSP90 before ER α degradation.

The energetics of XH binding to ER α was measured using ITC with GS, one of the major ingredients of XH. The binding reaction was exothermic and exhibited an ITC curve indicating the binding reaction of GS and ER α . The binding curve of GS and ER α was similar to that of estradiol and ER α . As the ER α protein was expressed by *Escherichia coli*, the physiological activities and functions of the recombinant estrogen receptor were different from those of ER α expressed by eukaryotes. In addition, the ER α protein and the compounds were not 100% pure. The results may not accurately show the affinity for these factors. However, the similarity of the binding curve of GS and ER α to that of the natural ligand (estradiol) and ER α indicates that GS has a high affinity for ER α .

As the major ingredients of XH could bind to both ER α and HSP90, we questioned whether HSP90 was involved in XH-triggered ER α degradation. There were three pieces of evidence. Firstly, several ingredients in XH had high affinity with HSP90 (Fig. 5). and XH promoted the dissociation of ER α and HSP90 prior to ER α degradation (Fig. 7A, B). Moreover, downregulation of the HSP90 clients Raf, Akt, Erk, HER2, ErbB3 was triggered by XH and its

Fig. 9. Overview of the mechanisms underlying the anti-breast cancer effects of XH. XH achieved its anti-cancer effects via promoting the disassociation of ER α and HSP90, causing the degradation of ER α and blocking the transportation of ER α to the nucleus. XH also caused the dissociation of ER α and other oncoproteins via binding to HSP90.

components (Fig. 7C, D). Moreover, the different inhibitory effects of the major predicted ingredients in the four breast cancer cell lines also helped explain the dual targeting of ER α and HSP90 of XH in some ways, as the ingredients, predicted to target only ER α , only had inhibitory effects on ER+ cell lines but not others. In contrast, the ingredients, predicted to bind to HSP90 exhibited inhibitory effects on all the four breast cancer cell lines (Fig. 7). Therefore, we propose that HSP90 is involved in XH-induced ER α degradation.

Taken together, we mapped the hub targets to the canonical signaling pathways according to the results of the network-based systems and molecular-biological analyses. As showed in Fig. 9, XH mediated its anti-cancer effects by promoting the disassociation of ER α and HSP90, causing the degradation of ER α and blocking the transport of ER α to the cell nucleus. XH also caused the dissociation of ER α and other oncoproteins via binding to HSP90.

In conclusion, we found that XH, which has been used for breast cancer treatment since 1740, has antiestrogenic effects in ER+ breast cancer by causing the degradation of ER α , antagonizing its effect and disrupting nuclear localization, similar to the mechanism of action of fulvestrant. More importantly, XH also caused the dissociation of ER α and other oncoproteins via binding to HSP90.

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Disclosure Statement

The authors declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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