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Rhizoma Amorphophalli Inhibits TNBC Cell Proliferation, Migration, Invasion and Metastasis Though the PI3K/Akt/mTOR Pathway

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Abstract

*Ethnopharmacological relevance:* Triple-negative breast cancer (TNBC) often presents with a high histological grade and high malignancy, which greatly contribute to patient morbidity and mortality. *Rhizoma Amorphophalli* exhibits many biological and pharmacological activities, but its potential as a therapeutic agent for the treatment of metastatic TNBC patients remains poorly understood.

*Aim of the study:* The aim of this study was to determine whether *Rhizoma Amorphophalli* inhibits metastasis in the human TNBC MDA-MB-231 cell line.

*Materials and methods:* CCK-8 and colony formation assays were adopted for the analysis of cell activity and cell proliferation, respectively. Flow cytometry was used for cell cycle analysis. Wound healing and transwell assays were performed to assess cell migration and invasion, respectively. PI3K/Akt/mTOR signaling pathways were analyzed through western blotting. Breast cancer cell metastasis to the lung in a xenograft model was evaluated by *in vivo* fluorescence imaging. A GC-MS analysis was performed to determine the main components of the petroleum ether fraction from the ethanol extract of *Rhizoma Amorphophalli* (abbreviated RhA).

*Results:* RhA significantly reduced breast cancer cell viability and proliferation. The flow cytometry analysis indicated that RhA induced MDA-MB-231 cell arrest at the S phase. Additionally, RhA decreased MDA-MB-231 cell migration and invasion and inhibited the PI3K/Akt/mTOR signaling pathway. In addition, mice treated with RhA exhibited a significant reduction in tumor infiltration and a decrease in breast cancer cell metastasis to the lung. The GC-MS analysis results showed that RhA contained a
large number of unsaturated fatty acids, such as octadecadienoic acid (linoleic acid),
onadecatrienoic acid (linolenic acid), and oleate, which might represent the
anticancer components of the extract.

Conclusions: The results of this study suggest that RhA has potential as a therapeutic
candidate for metastatic TNBC treatment.

Keywords: tumor metastasis; Rhizoma Amorphophalli; triple-negative breast cancer;
PI3K/Akt pathway; mTOR pathway

Abbreviations
CAM, complementary and alternative medicine; CCL21, chemokine (C-C motif)
ligand 21; CCR7, chemokine (C-C motif) receptor 7; CXCR4, C-X-C chemokine
receptor type 4; GC-MS, gas chromatography-mass spectrometry; Her2, human
epidermal growth factor receptor 2; LA, luminal A; LB, luminal B; mTOR,
mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; RhA,
petroleum ether fraction from the ethanol extract of Rhizoma Amorphophalli; SDF-1,
stromal cell-derived factor-1; TNBC, triple-negative breast cancer.
1. Introduction

Breast cancer is the most common malignancy that develops in women and is responsible for the highest rate of cancer-associated death in women. In recent years, global molecular analyses have revealed four main distinct subgroups of human breast tumors: luminal A (LA), luminal B (LB), human epidermal growth factor receptor 2 (Her2)-overexpressing and triple-negative breast cancer (TNBC) (Koboldt et al., 2012). Although TNBCs comprise only a small percentage of all breast cancers diagnosed (10-24%), they behave aggressively and are unresponsive to targeted adjuvant therapy. Patients who are diagnosed with TNBC are younger, tend to develop larger tumors and have an increased likelihood of distant metastasis and death within five years of diagnosis (Carey et al., 2010). In contrast to non-TNBCs, TNBC often exhibits a high histological grade and malignancy and preferentially metastasizes to the lung, liver and brain (Smid et al., 2008).

Patients with TNBC are often treated exclusively with conventional chemotherapy and do not benefit from targeted therapies due to the lack of estrogen receptors, progesterone receptors and Her2 receptors in the tumor (Albeck and Brugge, 2011). Although several small-molecule inhibitors targeting other oncogenic drivers, such as dasatinib (Src inhibitor), cetuximab (EGFR inhibitor), bevacizumab (vascular endothelial growth factor inhibitor) and olaparib (poly [ADP-ribose] polymerase inhibitor), are currently in clinical trials for TNBC patients, the identification of relevant molecular targets in TNBC remains a critical challenge (Toft and Cryns,
TNBCs represent a major clinical problem, and relevant targeted therapies are currently not available.

Accumulating data have indicated that complementary and alternative medicine (CAM) has a beneficial effect in the treatment of several types of cancers (Horneber et al., 2012; Wanchai et al., 2010; Zhai et al., 2013; Ling et al., 2014). Breast cancer survivors exhibit a strong tendency to use CAM treatments and have shown particular interest in natural product supplements and herbal remedies. Up to 60% of breast cancer survivors use herbal treatments, despite expressing concerns that their safety and efficacy are not well established (Matthews et al., 2007). Our previous study indicated that human TNBC cells (MDA-MB-231 or MDA-MB-231BO) are inhibited by extracts from Curcuma zedoaria, Psoralea corylifolia or Cnidium monnieri (Wu et al., 2013; Gao et al., 2014; Wu et al., 2017). Rhizoma Amorphophalli, a perennial herb tuber of the family Araceae and genus Amorphophallus that is also known as Amorphophallus konjac, is mainly distributed in tropical regions in Asia and Africa. Dried corms of Rhizoma Amorphophalli have been widely used in Chinese herbal prescriptions for the treatment of conditions such as obesity, diabetes, arterial sclerosis and cancers (Hua et al., 2007; Won et al., 2008). In China, many Chinese herbal formulas containing Rhizoma Amorphophalli have been used clinically as an adjuvant in the treatment of advanced or metastatic breast cancer, hepatoma, gastric cancer and pancreatic cancer patients. Pan et al. demonstrated that Rhizoma Amorphophalli induces gastric cancer and hepatoma cell apoptosis (Chen et al., 2008; Pan et al., 2010). A previous study also indicated that konjac glucomannan from Rhizoma Amorphophalli inhibits the growth of HeLa cells (He, 2014). In addition,
Chen et al. found that *Rhizoma Amorphophalli* inhibits hepatoma cell growth in mice (Chen et al., 2009). However, the inhibitory effect of *Rhizoma Amorphophalli* in breast cancer metastasis has not been identified. Therefore, the aim of the preclinical study described in this manuscript was to determine whether *Rhizoma Amorphophalli* inhibits metastasis in the human TNBC MDA-MB-231 cell line.

2. Materials and Methods

2.1. Materials

RPMI 1640 medium, fetal bovine serum (FBS), phosphate-buffered saline (PBS), TRIzol, and SuperScript reverse transcriptase enzyme and buffer were purchased from Gibco-BRL Company (Grand Island, NE, USA). Dimethyl sulfoxide (DMSO), DEPC and propidium iodide (PI) were purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO, USA). The CCK-8 Kit was purchased from the Dojindo Molecular Technologies (Japan). Antibodies to E-cadherin, stromal cell-derived factor-1 (SDF-1), phosphatidylinositol 3-kinase (PI3K), Akt, phospho-Akt, mammalian target of rapamycin (mTOR) and phospho-mTOR were purchased from Cell Signaling Technology (Boston, MA, USA). Antibodies to chemokine (C-C motif) ligand 21 (CCL21), C-X-C chemokine receptor type 4 (CXCR4), and chemokine (C-C motif) receptor 7 (CCR7) were purchased from Abcam (Cambridge, MA, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

2.2. Plant material and extraction procedure

Dried corms of *Rhizoma Amorphophalli* were purchased from Shanghai Cambridge
Traditional Chinese Medicine Co., Ltd. (Shanghai, China). The identity of the plant was confirmed through source identification, morphological examination and microscopic identification in comparison to herbarium specimens by a senior Traditional Chinese Medicine pharmacist at our hospital.

The dried corms were pulverized and passed through a 40-mesh sieve. The sample (40 g) was weighed accurately, placed into a 100-mL flask containing 80 mL of 95% ethanol and allowed to soak overnight. The sample was extracted twice times by heating under reflux. The ethanol extracts were filtered and concentrated under reduced pressure to obtain a residue, and the residue was then extracted with petroleum ether. The petroleum ether fraction from the ethanol extract of Rhizoma Amorphophalli (abbreviated RhA) was dried using a rotary evaporator, and RhA was dissolved in DMSO to prepare a 10 mg/mL stock solution. The stock solution was stored at -20 °C until use.

2.3. Gas chromatography-mass spectrometry (GC-MS) assay

An Agilent 7890A gas chromatograph/5975 mass selective detector system with an HP-5 capillary column (60 m×0.32 mm×0.25 mm) (Agilent Technologies) was used for the GC-MS analysis. Briefly, 1 μL of the extract sample was injected at a temperature of 260 °C into the split/splitless injector. Helium was used as the carrier gas at a flow rate of 1.2 mL/min, and the split ratio was 50:1. The oven temperature program was as follows: the initial temperature of 50 °C was held for 1 min, increased at a rate of 5 °C/min to 140 °C, increased at a rate of 10 °C/min to 240 °C, and maintained at 240 °C for 10 min. The analytes were detected in full scanning mode.
The MS acquisition parameters included scanning from \( m/z \) 30 to 400 in the electron impact (EI) mode for routine analysis. The electron energy was set to 70 eV, and the source temperature was set to 260 °C.

### 2.4. Cells and cell culture

Human TNBC MBA-MB-231 cells (a human breast adenocarcinoma cell line) were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The MDA-MB-231BO cell line is an exclusive bone metastatic subclone of MDA-MB-231 that was acquired through repeated injection of MDA-MB-231 cells into the left ventricle and subsequent isolation of the tumor cells from bone metastasis lesions, as described previously (Wu et al., 2017). MBA-MB-231 and MDA-MB-231BO cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 40 mg/ml gentamicin. The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. GFP-labeled MDA-MB-231 cells were enriched by fluorescence-activated cell sorting.

### 2.5. CCK-8 assay

Cell viability was determined using the cell counting kit-8 (CCK-8) reagent according to our previous work (Gao et al., 2014). Briefly, MDA-MB-231 or MDA-MB-231BO cells were incubated for 24 h with the indicated concentrations of RhA or epirubicin petroleum ether extract or with DMSO as the vehicle. The cell viability was determined through CCK-8 assays according to the manufacturer’s instructions, and the absorbance at 450 nm was measured with a microplate reader (Synergy 2
Multi-Mode Microplate Reader; BioTek, Winooski, VT, USA) and is expressed as a percentage of the control level. The mean optical density (OD) values from triplicate wells for each treatment were used as the index of cell viability.

2.6. Colony formation assay

The cells were seeded in six-well plates at a density of 500 cells per well, and after 10 days, colonies with a diameter greater than 0.05 mm were counted.

2.7. Cell cycle analysis

A cell cycle analysis was performed according to our previous work (Gao et al., 2014). Briefly, MDA-MB-231 cells were plated in 24-well plates and maintained in 10% FBS at a density of 1×10^6 cells for 24 h. The cells were then exposed to RhA (300 μg/mL) or epirubicin (1 μg/mL) for 24 h, harvested via trypsinization and pelleted by centrifugation. The pellets were resuspended in PBS containing 50 μg/mL PI, 0.1% Triton X-100, and 0.1% sodium citrate and then measured using a flow cytometer (FACS Canto II, Becton Dickinson, USA).

2.8. Western blotting

MDA-MB-231 cells were treated with or without RhA, and the total cell protein was harvested by centrifugation at 14,000 xg and 4 °C for 10 min. Quantification of the protein concentration and western blot assays were performed as described previously with slight modifications (Wu et al., 2017). Briefly, equal amounts of protein (20 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes. The
membranes were blocked with 5% BSA and then incubated with the appropriate primary antibodies overnight at 4 °C. Two imaging analysis experts independently analyzed and quantified the immunoblotting data using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Any quantitative results generated by these two experts that differed substantially (>5% discrepancy) were further analyzed until an agreement was reached. The data were then normalized to the vehicle group. The values for each group were subsequently transformed to log2 ratios, and p values were calculated using a t-test.

2.9. Wound healing assay

Wound healing assays were performed as described previously (Wu et al., 2017). Briefly, 8 × 10^4 cells were seeded in each well of a six-well plates, and when the cellular confluence reached approximately 90%, a 200-μl pipette tip was used to create a wound in the confluent cell layer. The wells were then rinsed with medium to remove any free-floating cells and debris, and medium without serum containing various concentrations of RhA was added. Within 24 or 48 h after the scrape line was made, wound healing was observed, and representative scrape lines were imaged. Each assay was conducted in triplicate. The migration rate was calculated as follows:

Migration rate (%) = (Scratch distance at 0 h - Scratch distance at indicated time) / Scratch distance at 0 h × 100.

2.10. Transwell assay

Transwell assays were performed as described previously with slight modifications (Wu et al., 2017). Briefly, 3 × 10^4 cells were seeded with or without RhA into the
upper chamber of 24-well transwell chambers (Corning Inc., Corning, NY, USA) separated by a polycarbonate filter coated with 50 μg/ml collagen IV. Serum-free medium was added to the upper chamber, and 10% FBS medium was added to the bottom chamber. After 24 or 48 h, the cells on the top side of the inserts were scraped off, and the transwell filters were stained with crystal violet and examined under an inverted microscope. The cells in triplicate wells that had migrated within 24 or 48 h were quantified.

2.11. Animals and xenograft model procedure

Female nude (BALB/c nu/nu) mice (20 ± 2 g) were housed in a temperature-controlled (24 ± 2 °C) room with a regular 12-h light/dark cycle. After one week of acclimatization in a specific pathogen-free (SPF) environment, the animals were randomly assigned to one of several experimental groups. All animals had free access to water and food until the day before the experiment. All experiments were performed in accordance with national regulations for animal experimentation and approved by the Institutional Animal Care and Use Committee (IACUC) of Zhejiang Chinese Medical University.

The mice received an intravenous injection of GFP-labeled MDA-MB-231 cells (4×10⁶ cells/mouse). The mice were then randomly divided into three groups (n = 10 per group): one group received RhA (4.25 g/kg, p.o., q.d.), another group received epirubicin (7.5 mg/kg, i.p., q.w.), which was used as a positive control (Zhou et al., 2017), and the last group was administered the vehicle. These treatments were administered for four weeks. The dose of RhA was converted from the equivalent clinical dose. The clinical doses of RhA and epirubicin were 30 g/day and 33.3
mg/m²/week, respectively. Before the end of the treatment, breast cancer cell metastasis was evaluated by in vivo fluorescence imaging. The mice were sacrificed at the end of the treatment period, and metastatic lung lesions observed by fluorescence imaging were sectioned and used for hematoxylin and eosin (H&E) staining (200× magnification).

2.12. Fluorescence imaging in vivo

Fluorescence imaging was performed according to a previous study (Wang et al., 2015). Briefly, the animals were imaged using an IVIS Lumina II in vivo imaging system (Caliper Life Sciences, USA) at the end of the treatment period using the manufacturer’s recommended procedures and settings. We focused on the fluorescence signals in the lungs.

2.13. Histological evaluation

After the lungs with metastatic lesions were identified through fluorescence imaging analyses, the sample tissues were dissected, sectioned, and stained with H&E. Histological evaluation of the metastatic lesions was performed as described previously (Wu et al., 2017). Briefly, the cancer cells were identified, and the percentage of cancer cells per high-power field-of-view (400× magnification) was calculated. All histological sections were examined by two independent pathologists. Sections that received substantially different scores from the two pathologists (>5% discrepancy) were reviewed again until a consensus was reached.
2.14. Statistical analysis

All results are presented as the means ± standard deviations. Two-tailed analysis of variance (ANOVA) followed by Dunnett’s post hoc test and Fisher’s test were used to determine statistical significance. A p value < 0.05 was considered significant for all tests.

3. Results

3.1. RhA inhibits the viability and proliferation of TNBC cells

To determine the effects of RhA on cell viability, we performed CCK-8 assays to measure the effects of RhA on the viability of breast cancer MDA-MB-231 and MDA-MB-231BO cells. As shown in Figure 1 A/C, MDA-MB-231 cells treated for 24 h with RhA at a concentration greater than or equal to 100 μg/ml displayed significant inhibition of cell viability in vitro in a drug dosage-dependent manner. In another TNBC cell line, MDA-MB-231BO, RhA also dosage-dependently inhibited cell viability (Figure 1 B/C). The IC₅₀ values of RhA in MDA-MB-231 and MDA-MB-231BO cells were approximately 406 and 458 μg/ml, respectively. We then further analyzed the cell growth curve and found that RhA continuously inhibited MDA-MB-231 and MDA-MB-231BO cell viability (Figure 1 E). Additionally, to further examine the effect of RhA on MDA-MB-231 cell proliferation, we performed colony formation assays. As shown in Figure 1 F, concentrations of RhA greater than or equal to 200 μg/ml significantly inhibited the proliferation of breast cancer MDA-MB-231 cells. These data confirm that RhA inhibits breast cancer cell proliferation and reduces the viability of human TNBC cells.
3.2. RhA suppresses the migration and invasion of MDA-MB-231 cells

To determine the role of RhA in cellular migration, MDA-MB-231 cells were treated with a non-toxic concentration (50 μg/ml) of RhA for 24 h. Following treatment, the ability of MDA-MB-231 cells to migrate into wounds created by scratching confluent cells with a pipette tip was determined. As shown in Figure 2 A/C, MDA-MB-231 cells treated with RhA displayed significant delays in wound closure resulting from diminished cell migration. Quantification of wound size revealed that RhA inhibited wound closure by an average of 30% compared with that observed in the vehicle-treated cells. Additionally, we performed a transwell assay to assess the ability of RhA to inhibit cell invasion. RhA significantly reduced the number of invaded cells (Figure 2 B). The inhibition of breast cancer cell invasion by RhA was higher than 10% (Figure 2 D).

3.3. RhA induces S-phase arrest in MDA-MB-231 cells

We further investigated the effect of RhA on cellular migration. MDA-MB-231 cells were treated with RhA for 24 h and then subjected to cell cycle analysis using flow cytometry. As shown in Figure 3, RhA significantly reduced the proportion of cells in the G1 and G2/M phases and substantially increased the number of cells in the S phase. These results indicate that RhA induces S-phase arrest in MDA-MB-231 cells.

3.4. RhA regulates the expression of cell adhesion factor and chemokines

Chemokines and adhesion family proteins, such as E-cadherin, CCR7, SDF-1, CCL21 and CXCR4, are strongly linked to tumor recurrence and metastasis (Gupta and
Massagué, 2006). Therefore, we tested the expression of these proteins in MDA-MB-231 cells after RhA treatment through western blotting and found that RhA significantly increased E-cadherin and reduced CCR7 and CXCR4 expression (Figure 4).

3.5. RhA inhibits the PI3K/Akt/mTOR signal pathway

Abnormal activation of the phosphatidylinositol-3 kinase (PI3K)/Akt and mTOR (PI3K/Akt/mTOR) pathway is implicated in both the pathogenesis of breast cancer and the development of resistance to anticancer therapies (Cairns et al., 2011; Shaw and Cantley, 2006). Several important members of the PI3K/Akt/mTOR signaling pathway were analyzed by western blotting. RhA significantly decreased PI3K expression (Figure 5 A/B). The degree of Akt and mTOR phosphorylation was also apparently inhibited by RhA (Figure 5 A/B). To confirm the inhibitory role of the PI3K/Akt/mTOR signaling pathway, we used PI3K/Akt/mTOR pathway inhibitors (LY294002/deguelin/rapamycin) to demonstrate that the RhA-mediated changes in the pathway suppressed the migration of breast cancer MDA-MB-231 cells. As shown in Figure 5 C-E, RhA, deguelin and rapamycin separately reduced breast cancer cell migration. The inhibition of cell migration by RhA was not significantly increased by LY294002, deguelin or rapamycin. In other words, additional inhibition of the PI3K/Akt/mTOR signaling pathway by LY294002, deguelin or rapamycin did not increase the inhibition obtained with RhA. These results indicate that RhA inhibits cell migration though the PI3K/Akt/mTOR signaling pathway in MDA-MB231 breast cancer cells.
3.6. **RhA inhibits breast cancer cell metastasis in mice**

MDA-MB-231 cells are known to metastasize aggressively in xenograft models. We intravenously injected GFP-labeled MDA-MB-231 cells into mice to monitor breast cancer cell metastasis. After four weeks of treatment with RhA, the fluorescence intensity of tumor metastases in the lung was suppressed by 40% on average compared with the fluorescence in the vehicle-treated mice (Figure 6 A/B). We then evaluated the lungs for metastatic lesions histologically and calculated the number of tumor cells present in the lesions. RhA administration resulted in a significant reduction in tumor infiltration and an average 25% decrease in the percentage of tumor cells in metastatic lesions compared with vehicle-treated mice (Figure 6 C/D). However, we found that RhA did not obviously inhibit body weight gain in tumor-bearing mice after four weeks of treatment (Figure 6 E).

3.7. **Main components of RhA determined by GC-MS**

To clarify the main components of RhA, a GC-MS assay was adopted. Comparison of the results with a commercial database, identified 31 compounds (Figure 7 A). According to the normalization method, the detectable components of RhA are shown in Table 1. The sum of the first 13 components of RhA is more than 90% (Figure 7 B). Acids can react with alcohols to form esters when heated, and dry *Rhizoma Amorphophalli* soaked overnight in ethanol was extracted by heating under reflux. Therefore, ethyl esters detected by GC-MS are likely fatty acids (FAs). For example, octadecadienoic acid reacts with alcohols to form octadecadienoic acid ethyl ester. Therefore, the FA content is greater than 34.6% (Figure 7 C). Further studies are needed to confirm the components of the petroleum ether extract of *Rhizoma*...
4. Discussion

*Amorphophalli.*

*Rhizoma Amorphophalli* is used for a variety of applications in traditional Chinese medicine and exhibits many pharmacological and biological activities. However, the therapeutic role and regulatory mechanism of *Rhizoma Amorphophalli* in breast cancer metastasis remain poorly understood. In the present study, RhA induced significant decreases in cell proliferation and suppressed the migration and invasion of breast cancer cells. RhA also inhibited the PI3K/Akt/mTOR pathway in breast cancer MDA-MB-231 cells. In addition, we found that RhA induced S-phase arrest in the breast cancer MDA-MB-231 cell line. Furthermore, we demonstrated that mice treated with RhA exhibited a significant reduction in tumor infiltration and a decrease in the percentage of tumor cells in metastatic lung lesions. Moreover, in tumor-bearing mice, RhA significantly reduced breast cancer cell metastasis to the lung.

Compared with normal epithelia, cancer cells almost invariably show diminished intercellular adhesiveness (Cavallaro and Christofori, 2004). In many instances, epithelial tumors lose E-cadherin-mediated adhesions as they progress toward malignancy (Gupta and Massagué, 2006). E-cadherin is mainly expressed at the cell membrane in breast cancer cells primarily expressing the wild-type E-cadherin gene sequence, such as MCF-7, MDA-MB-361, MDA-MB-468, and T47D cells, and is expressed at relatively low levels in breast cancer cells, such as BT549, Hs578T, and MDA-MB-231 cells (Van et al., 2001). These differences might alter the metastatic
In the present study, we found that RhA increases E-cadherin expression in MDA-MB-231 cells. This result suggests that E-cadherin expression might be a factor in the inhibitory effect of RhA on breast cancer cell metastasis.

Tumor cell migration and metastasis share many similarities with leukocyte trafficking, which is critically regulated by chemokines and their receptors (Müller et al., 2001). In breast cancer cells, signaling through CXCR4 or CCR7 mediates actin polymerization and pseudopodia formation and subsequently induces chemotactic and invasive responses (Cabioglu et al., 2005). In vivo, neutralizing the interactions of CXCL12/CXCR4 leads to a significant inhibition of lung metastasis (Müller et al., 2001). Chemokines and their receptors play a critical role in determining the metastatic destination of tumor cells. In the current study, breast cancer MDA-MB-231 cells treated with RhA exhibited significant reductions in CCR7 and CXCR4 expression but not in CCL21 and SDF-1 expression. The results of the wound healing and transwell analyses also showed that RhA significantly decreased cell migration and invasion. These results indicate that the inhibitory effect of RhA on CCR7 and CXCR4 expression might decrease cell invasion and migration.

The PI3K/Akt/mTOR pathway mediates multiple cellular functions critical to tumor initiation, progression, and outcome, including growth, proliferation, metabolism, motility, migration, invasion, angiogenesis, and survival (Shaw and Cantley, 2006). Frequent aberrations of this pathway have been implicated in breast cancer development and progression (Mcauliffe et al., 2010). To gain insight into the
mechanisms involved in the RhA treatment-mediated inhibition of migration and invasion, we further investigated the effect of RhA on the PI3K/Akt and mTOR pathways. Western blotting analyses revealed that treatment with RhA leads to significant decreases in PI3K protein expression. Although RhA did not regulate total Akt and mTOR protein expression, it significantly suppressed the phosphorylation of Akt and mTOR in MDA-MB-231 cells. We also found that RhA inhibits the migration of MDA-MB231 breast cancer cells through the PI3K/Akt/mTOR signaling pathway. These results indicate that the inhibitory effect of RhA on breast cancer depends on the suppression of the PI3K/Akt/mTOR pathway.

Although FAs are essential constituents of all biological membrane lipids and important substrates for energy metabolism, saturated fatty acids (SFAs) in the diet or synthesized de novo might modulate molecular mechanisms relevant to carcinogenesis (Forman and Mahabir, 2010). In addition, fatty acid synthase works as a previously unrecognized metabolic intermediate of oncogenesis, linking energy, anabolism and malignant transformation via the PI3K/Akt and mitogen-activated ERK kinase (MEK)-ERK pathways (Menendez and Lupu, 2007). However, unsaturated fatty acids (USFAs), including ω-3 and ω-6 FAs and α-linolenic acid, are beneficial for preventing and treating cancer (Johanning and Lin, 1995). Through GC-MS analysis, we found that RhA contains a greater number of USFAs, such as octadecadienoic acid (linoleic acid), octadecatrienoic acid (linolenic acid), and oleate. Recent studies have provided strong evidence that many dietary compounds, including conjugated linoleic acid, induce apoptosis and suppress cell cycle progression due to suppression of the PI3K/Akt and ERK signaling cascades in
different types of cancer (Cho et al., 2009; Kim et al., 2003; Lee et al., 2006).

Additionally, the anti-carcinogenic activity of α-linolenic acid against mammary cancer is associated with a reduction in the expression of the oncogenes fatty acid synthase and human epidermal growth factor receptor 2. The results of the present study suggest that the main anticancer components of RhA are likely USFAs. Moreover, RhA also contains a considerable proportion of SFAs, such as hexadecanoic acid (palmitic acid). Therefore, further studies are needed to confirm the active principle of RhA in breast cancer metastasis.

Taken together, the findings of this study show that the inhibitory effects of RhA are attributed to the inhibition of cell viability and proliferation, the induction of cell cycle arrest, and the blockade of migration and invasion, which might be mediated via perturbations in the PI3K/Akt/mTOR signaling pathway (Figure 8). Additionally, RhA significantly reduces breast cancer cell metastasis to the lung in tumor-bearing mice. Therefore, RhA might be useful for preventing TNBC cell metastasis. Further studies are required to screen the main active components of RhA against metastatic breast cancer.

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Conflict of Interest

The authors declare no potential conflicts of interest.

Figure Legends:

Figure 1. RhA inhibited the viability and proliferation of TNBC cells. (A) Breast cancer MDA-MB-231 cells were incubated with the indicated concentration of RhA or epirubicin for 24 h and then assessed through CCK-8 assays. The results are expressed as the means ± SDs of three experiments (* p < 0.05 compared with the vehicle group, as determined by ANOVA). (B) Same as (A) with the exception that MDA-MB-231BO cells instead of MDA-MB-231 cells were examined. (C) The inhibitory effect of different concentrations of RhA on MDA-MB-231 and MDA-MB-231BO cells treated for 24 h was assessed through CCK-8 assays. (D) The LD₅₀ value of RhA was determined using CCK-8 assays. The results are expressed as the means ± SDs of three experiments. (E) The cell growth curve was constructed from CCK-8 assay data. The concentrations of RhA and epirubicin were 300 μg/ml and 1 μg/ml, respectively. Left: MDA-MB-231 cells; Right: MDA-MB-231BO cells. The results are expressed as the means ± SDs of three experiments (* p < 0.05 compared with the vehicle group, as determined by ANOVA). (F) Quantitative results of colony formation (% of vehicle). The values are expressed as the means ± SDs of three experiments. The colony formation of vehicle-treated cells was set to 100%, and the values for the colony formation of RhA-treated cells are presented as percentages of vehicle colony formation (* p < 0.05 compared with the vehicle group, as determined by ANOVA).
Figure 2. Anti-invasion and anti-migration effects of RhA on breast cancer

MDA-MB-231 cells. Breast cancer MDA-MB-231 cells were treated with or without 50 μg/ml RhA for 24 or 48 h and then prepared for wound healing and transwell assays. (A) Representative images from wound healing assays of cells treated with RhA (50 μg/ml), epirubicin (1 μg/ml) or vehicle were obtained at 0, 24 and 48 h. Left: vehicle group; Middle: epirubicin group; Right: RhA group. (B) Representative images of transwell analysis of cells treated with RhA (50 μg/ml), epirubicin (1 μg/ml) or vehicle were obtained at 24 and 48 h. Left: vehicle group; Middle: epirubicin group; Right: RhA group. (C) The migration rate was quantified as the percentage of wound closure and expressed as the mean ± SD of three experiments (* p < 0.05 compared with the vehicle group, as determined by ANOVA). Migration rate (%) = (Scratch distance at 0 h - Scratch distance at indicated time) / Scratch distance at 0 h × 100. (D) The quantitative results of the transwell migration assay are expressed as the means ± SDs of three experiments (* p < 0.05 by ANOVA compared with the vehicle group, as determined by ANOVA).

Figure 3. RhA induced S-phase arrest in breast cancer cells. Cells were treated with or without 300 μg/ml RhA for 24 h and then prepared for cell cycle analysis. (A) Representative flow cytometry analysis data obtained after PI staining are shown. The apoptosis levels of cells treated with either RhA or vehicle were assessed at 24 h. Left: vehicle group; Middle: epirubicin group; Right: RhA group. (B) and (C) Quantitative results of cell cycle distribution determined through PI staining and flow cytometry analysis and are expressed as the means ± SDs of three experiments (* p < 0.05 compared with the vehicle group, as determined by ANOVA).
Figure 4. RhA regulated cell adhesion factor proteins and chemokines. (A) Representative images of protein expression detected by western blotting. Cells were treated with or without 300 µg/ml RhA for 24 h. Protein lysates were analyzed by western blotting. (B) Quantification of the protein expression results (log2-ratio vs. vehicle; means ± SDs). Scanning densitometry was used for the semiquantitative analysis, which was performed in comparison to the vehicle-treated group. The results are expressed as the means ± SDs of three experiments (* p < 0.05 compared with the vehicle group, as determined by ANOVA).

Figure 5. RhA inhibited the PI3K/Akt and mTOR signaling pathways. (A) Representative images of protein expression detected by western blotting. Cells were treated with or without 300 µg/ml RhA for 24 h. (B) Quantification of the protein expression results (log2-ratio, * p < 0.05 compared with the vehicle group, as determined by ANOVA). Scanning densitometry was used for the semiquantitative analysis. The results are expressed as the means ± SDs of three experiments. (C) Representative images of cell migration induced by RhA in MDA-MB-231 cells pre-treated with or without PI3K/Akt and mTOR inhibitors (LY294002, deguelin and rapamycin). After wounding, MDA-MB-231 cells were treated with LY294002 (10 µM), deguelin (1 µM) or rapamycin (1 µM) for 2 h and then incubated with or without 50 µg/ml RhA for another 22 h. Representative images were taken at 0 and 24 h. (D) Quantitative analysis results of cell migration from the wound healing experiments. Wound closure was quantified as the wound migration rate and is expressed as the means ± SDs of three experiments (* p < 0.05 compared with the vehicle group, as determined by ANOVA). (E) Quantitative analysis results of cell migration from the wound healing assays. The migration distance from the wound healing of
vehicle-treated cells was set to 100%, and the wound distance of the RhA-,
epirubicin- or PI3K/Akt/mTOR inhibitor-treated cells is presented as a percentage of
that of the vehicle group (* p < 0.05 compared with the vehicle group, as determined
by ANOVA).

**Figure 6. RhA inhibited breast cancer cell metastasis in mice.** Mice received an
intravenous injection of GFP-labeled MDA-MB-231 cells (4×10^6 cells/mouse). The
mice were then randomly divided into three groups (n = 10 per group): one group
received RhA (4.25 g/kg, p.o., q.d.), another received epirubicin (7.5 mg/kg, i.p., q.w.),
and the third received the vehicle. The treatment was administered once daily for four
weeks. Before the end of the treatment period, breast cancer cell metastasis was
evaluated by *in vivo* fluorescence imaging. The mice were sacrificed at the end of the
treatment period, and metastatic lung lesions observed by fluorescence imaging were
sectioned and subjected to H&E staining (magnification: 200×). (A) Representative
fluorescence images obtained at the end of the treatment period. Left: vehicle group;
Middle: epirubicin group; Right: RhA group. (B) Quantitative analysis of the
bioluminescence of the tumor burden in lung lesions at the end of the treatment period
(* p < 0.05 compared with the vehicle group, as determined by ANOVA). (C)
Representative images of H&E staining of lung metastases. Breast cancer cells were
marked by an arrow. Left: vehicle group; Middle: epirubicin group; Right: RhA group.
(D) Quantitative histomorphometry measurement of the tumor burden in lung lesions
through H&E staining (* p < 0.05 compared with the vehicle group, as determined by
ANOVA). (E) Body weight changes of tumor-bearing mice after four weeks of RhA
administration (* p < 0.05 compared with the vehicle group, as determined by
ANOVA).
Figure 7. Main components of RhA determined by GC-MS. The 31 identified compounds are shown in Table 1. (A) Total ion chromatograph of RhA obtained by GC-MS. (B) The main components of RhA were analyzed quantitatively via area normalization. (C) Primary classification of the measurable components determined by GC-MS.

Figure 8. Schematic representation of the signaling pathways likely involved in RhA-mediated breast cancer cell metastasis. RhA inhibits breast cancer cell growth, migration, and invasion and induced S-phase arrest in breast cancer cells. In addition, RhA increases E-cadherin expression and decreases the expression of chemokines (CCR7 and CXCR4). Additionally, RhA also inhibits the PI3K/Akt and mTOR signaling pathways in metastatic breast cancer MDA-MB-231 cells. In addition, RhA significantly reduces breast cancer cell metastasis to the lung in tumor-bearing mice.

References


Fig. 1
Fig. 2
Fig. 3

Fig. 4
Fig. 5
Fig. 6
Fig. 7
Fig. 8
Graphical Abstract

Table 1. The main components of RhA determined by GC-MS.

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