Journal of Ethnopharmacology 211 (2018) 89–100

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Rhizoma Amorphophalli inhibits TNBC cell proliferation, migration, invasion and metastasis through the PI3K/Akt/mTOR pathway

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ARTICLE INFO

Keywords:
Tumor metastasis
Rhizoma Amorphophalli
Triple-negative breast cancer
PI3K/Akt pathway
mTOR pathway

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), octadecatrienoic 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.

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

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

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http://dx.doi.org/10.1016/j.jep.2017.09.033
Received 5 July 2017; Received in revised form 15 September 2017; Accepted 24 September 2017
Available online 27 September 2017
0378-8741/

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, 2011). 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-23 l or MDA-MB-231BO) are inhibited by extracts from *Curcuma zedoaria*, *Psoralea corylifolia* or *Cnidium monnieri* (Wu et al., 2013, 2017; Gao et al., 2014). *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 and Liu, 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* 30–400 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% CO_2. GFP-labeled MDA-MB-231 cells were enriched by fluorescence-activated cell sorting.

2.5. CCK-8assay

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 DSMO 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