Marine algae *Sargassum horneri* bioactive factor suppresses proliferation and stimulates apoptotic cell death in human breast cancer MDA-MB-231 cells in vitro

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Abstract

Marine algae *Sargassum horneri* (*S. horneri*) bioactive factor has been shown to possess anabolic bone effects due to stimulating osteoblastic bone formation and suppressing osteoclastogenesis. However, the effects of *S. horneri* on cancer cell bone metastasis have not been investigated. This study was undertaken to determine the effects of *S. horneri* bioactive component on human breast cancer MDA-MB-231 bone metastatic cells in vitro. Proliferation of MDA-MB-231 cells was suppressed by culture with *S. horneri* active component (less than 3000 molecular weight; 10–200 μg/ml) for 5 and 10 days. *S. horneri* active component was suggested to inhibit G1 and G2/M phase cell cycle arrest in MDA-MB-231 cells using inhibitors of cell-cycle arrest. Moreover, *S. horneri* active component stimulated apoptotic cell death. This effect was prevented in the presence of caspase-3-inhibitor. Thus, *S. horneri* active component was found to suppress cell proliferation and stimulate apoptotic cell death in human breast cancer MDA-MB-231 bone metastatic cells in vitro, demonstrating an anticancer effect.

Introduction

Breast cancer is by far the most common cancer in women worldwide, and it is associated with a variety of lifestyle choice, such as obesity, later onset of first childbirth, and the use of hormone replacement therapy [1]. Breast cancer still remains the second cause of cancer death in the developed world. Breast cancer bone metastasis occurs in 70–80% of patients with advanced breast cancer, leading to severe pathological bone fractures, pain, hypercalcemia, and spinal cord and nerve-compression syndromes, which are a common cause of morbidity and mortality. Chemoprevention is the use of drugs, vitamins, food supplements, vaccines, or other agents to reduce the risk, delay of the development or recurrence of cancer [1,2].

Among marine algae of *Undaria pinnatifida*, *Sargassum horneri*, *Eisenia bicyclis*, *Cryptonemia semitziana*, *Gelidium amansii*, and *Ulva pertusa* Kjellman, which were gathered seasonally, *Sargassum horneri* (*S. horneri*) has been found to possess a unique anabolic effect on bone metabolism [3]. *S. horneri* extract possessed a stimulatory effect on osteoblastic bone formation and an inhibitory effect on osteoclastic bone resorption in vitro, thereby increasing bone mass [4–7]. Intake of *S. horneri* extract was found to prevent bone loss in osteoporosis animal models and in healthy human [8–10]. Functional food factor *S. horneri* extract may be usefulness as an osteogenic factor in preventing osteoporosis in human subjects. Moreover, *S. horneri* active component may possess a suppressive effect on bone loss induced by breast cancer cell bone metastasis that is occurred extremely high in breast cancer patients [11–15]. However, this has not been investigated.

This study was undertaken to determine whether *S. horneri* active component possesses anticancer effects using human breast cancer MDA-MB-231 bone metastatic cells in vitro model. We found that *S. horneri* active component suppresses proliferation and stimulates apoptotic cell death of human breast cancer MDA-MB-231 bone metastatic cells. This finding suggests that the intake of *S. horneri* active component is a useful tool in the prevention and therapy of breast cancer bone metastasis.

Materials and methods

Materials

Dulbecco’s Modification of Eagle’s Medium (DMEM) with 4.5 g/L glucose, L-glutamine and sodium pyruvate and antibiotics (penicillin and streptomycin; P/S) were purchased from Invitrogen Corp. (Carlsbad, CA). Fetal bovine serum (FBS) was from HyClone (Logan, UT). Sodium butyrate, roscovitine, sulforaphane, caspase-3 inhivitor and other all reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

*S. horneri* active component

Marine algae *S. horneri* (*Sargassum horneri* (Turner) C. Agardh) was seasonally gathered from the coast at Shimoda (Shizuoka Prefecture, Japan) and Miyako (Iwate Prefecture, Japan), and it was freeze-dried and powered [4]. The gathered fresh marine algae were homogenized in distilled water and are centrifuged at 5500 g in a refrigerated 

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centrifuge for 10 minutes. The 5500 g supernatant fraction was pooled for freeze-drying. The powder of the water-solubilized extract was dissolved in ice-cold distilled water for use in the experiments. The water-solubilized extract from S. horneri was purified by the method of membrane fractionation to collect the active component less than 3000 MW [4].

**Human breast cancer MDA-MB-231-bone metastatic cells**

Human breast cancer MDA-MB-231 bone metastatic cells (MDA-MB-231) lack estrogen, progesterone and human epithelial growth factor type 2 (HER2) receptors [16], and are therefore considered as triple negative. They express high levels of the epithelial growth factor receptor (EGFR) and activation of this receptor and its downstream signaling events enhance migration, proliferation, invasion, and progression of the malignant phenotype of these cells [16]. We used the estrogen-independent bone-seeking triple negative human breast cancer MDA-MB-231 cells. This cell line was obtained from the American Type Culture Collection (Rockville, MD, USA).

**Cell proliferation**

Breast cancer MDA-MB-231 cells (1x10^5/ml per well) were cultured in a 24-well plate in DMEM containing 10% FBS and 1% P/S in the presence or absence of S. horneri active component (less than 3000 MW; 10, 25, 50, 100 or 200 μg/ml) for 5 and 10 days in a water-saturated atmosphere containing 5% CO2 and 95% air at 37°C. In separate experiments, MDA-MB-231 cells were cultured for 3 days in the presence or absence of S. horneri active component (50 μg/ml) with or without sodium butyrate (10 and 100 μM), roscovitine (10 and 100 nM) or sulforaphane (1 and 10 nM). After culture, cells were detached from each culture dishes and counted [17].

**Apoptotic cell death**

Breast cancer MDA-MB-231 cells (1x10^5/ml per well) were cultured using a 24-well plate in DMEM containing 10% FBS and 1% P/S in the absence of S. horneri component for 5 days when reached to confluence, and then the cells were cultured in the presence of S. horneri component (less than 3000 MW; 10, 25, 50, 100 or 200 μg/ml) for 2 days. In separate experiments, cells were culture for 5 days without S. horneri, and then cells were cultured for 2 days in the presence of S. horneri (50 μg/ml) with or without caspase-3 inhibitor (5 μM). After culture, the cells were detached from each culture dishes and counted [18].

**Cell counting**

Cells were detached from each culture dishes using 0.2% trypsin plus 0.02% EDTA in Ca²⁺/Mg²⁺-free PBS for 2 min at 37°C, cells were collected after centrifugation [17-19]. Cells were resuspended on PBS solution and stained with eosin. Cell numbers were counted under a microscope using a Hemocytometer plate. For each dish, we took the average of two countings. Cell number showed as number per well of plate.

**Statistical analysis**

Data are expressed as the mean ± standard deviation (SD). Statistical significance was determined using GraphPad InStat version 3 for Windows XP (GraphPad Software Inc. La Jolla, CA). Multiple comparisons were performed by one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons post-test for parametric data as indicated. Data indicated P<0.05 was considered statistically significant.

**Results**

*S. horneri* active component suppresses cell proliferation

Number of human breast cancer MDA-MB-231 bone metastatic cells was increased with periods of culture in the presence of FBS. Presence of *S. horneri* active component (less than MW 3,000; 10-200 μg/ml) was found to suppress proliferation in human breast cancer MDA-MB-231 cells cultured for 5 (Figure 1A) and 10 (Figure 1B) days *in vitro*. Thus, *S. horneri* active component was found to possess suppressive effects on cell proliferation.

Suppressive effects of *S. horneri* active component on proliferation in MDA-MB-231 cells were determined in the presence of various inhibitors that induce cell-cycle arrest *in vitro* (Figure 2). Cells were cultured for 3 days in the absence (Figure 2A) or presence (Figure 2B) of *S. horneri* active component (50 μg/ml) with or without butyrate (10 and 100 μM), roscovitine (10 and 100 nM) or sulforaphane (1 and 10 nM) [17]. Proliferation of MDA-MB-231 cells was suppressed in the presence of these inhibitors (Figure 2A). Suppressive effects of *S. horneri* active component on cell proliferation were not potentiated in the presence of these inhibitors (Figure 2B).

![Figure 1. S. horneri active component suppresses proliferation in human breast cancer MDA-MB-231 cells in vitro.](image-url)
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*S. horneri* active component are unknown. *S. horneri* active component has been shown to stimulate osteoblastogenesis and suppress osteoclastogenesis through antagonizing signaling pathway of nuclear factor (NF)-κB *in vitro* [4]. Signaling of NF-κB, which is enhanced in breast cancer cells, has been shown to promote osteolytic bone metastasis by inducing osteoclastogenesis via granulocyte-macrophage colony-stimulating factor (GM-CSF) [20].

Suppressive effects of *S. horneri* active component on proliferation of MDA-MB-231 cells were not seen in the presence of butyrate, roscovitine or sulforaphan that induce cell-cycle arrest. Roscovitine is a potent and selective inhibitor of the cyclin-dependent kinase cdc2, cdk2, and cdk5 [21]. Sulforaphane induces G2/M phase cell cycle arrest [22]. Butyrate induces an inhibition of G1 progression [17]. HCA was suggested to inhibit G1 and G2/M phase cell cycle arrest in MDA-MB-231 cells.

*S. horneri* active component was found to stimulate apoptotic cell death

**S. horneri** active component stimulates apoptotic cell death

Human breast cancer MDA-MB-231 cells were cultured for 5 days when reached to confluent, and then the cells were cultured for an additional 2 days in the presence of *S. horneri* active component (less than 3000 MW; 10-250 μg/ml). Cell number was decreased by addition of *S. horneri* active component (Figure 3A). Stimulatory effects of *S. horneri* active component (25 or 50 μg/ml) on cell death in MDA-MB-231 cells were blocked in the presence of caspase-3 inhibitor (5 μM) *in vitro* (Figure 3B). Thus, culture with *S. horneri* active component stimulated apoptotic cell death in breast cancer MDA-MB-231 cells *in vitro*.

**Discussion**

Marine algae *S. horneri* active component was found to suppress proliferation and stimulate apoptotic cell death in human breast cancer MDA-MB-231 bone metastatic cells *in vitro*. This finding demonstrates that *S. horneri* possesses an anticancer effect in MDA-MB-231 cells *in vitro*. This was the first time finding.

Suppressive effects of *S. horneri* active component on cell proliferation in MDA-MB-231 cells may be related to regulation of intracellular signaling pathways *in vitro*, although anticancer effects of

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**Figure 2.** Effect of *S. horneri* active component on proliferation in human breast cancer MDA-MB-231 cells in the presence of various inhibitors that induce cell-cycle arrests *in vitro*. Cells were culture for 3 days in the absence (A) or presence (B) of *S. horneri* active component (less than 3000 MW; 50 μg/ml) with or without butyrate (10 and 100 μM), roscovitine (10 and 100 nM) or sulforaphane (1 and 10 mM). After culture, the number of attached cells on dish was counted. Data are presented as mean ± SD of 2 replicate wells per data set using different dishes and cell preparation. *p*<0.001 versus control (white bar). 1 way ANOVA, Tukey-Kramer post test.

**Figure 3.** *S. horneri* active component stimulates apoptotic cell death in human breast cancer MDA-MB-231 cells *in vitro*. A: Cells were cultured for 5 days when reached to confluent, and then the cells were cultured for an additional 2 days in the presence of *S. horneri* active component (less than 3000 MW; 10-250 μg/ml). B: Cells were cultured for 5 days when reached to confluent, and then the cells were cultured for an additional 2 days in the presence of *S. horneri* active component (less than 3000 MW; 25 or 50 μg/ml) with or without caspase-3 inhibitor (5 μM). After culture, the number of attached cells on dish was counted. Data are presented as mean ± SD of 2 replicate wells per data set using different dishes and cell preparation. *p*<0.001 versus control (grey bar). 1 way ANOVA, Tukey-Kramer post test.
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Effective compounds in S. horneri active components, which suppress proliferation and stimulate apoptotic cell death in MDA-MB-231 cells, have not identified. However, we found the existence of 4 chemicals in S. horneri active components (less than MW 3000) using the analysis with liquid chromatography mass spectrometry system (LCMS-FT-TOF; Shimadzu, Kyoto, Japan). These chemicals were identified as 1,3,5-tris(oxolan-2-ylmethyl)-1,3,5-triazinane (MW 339), 5-phenyl-2-[2-(5-phenyltetrazol-2-yl)ethyl]tetrazole (MW 318), 3-(hexadecylamino)propane-1,2-diol (MW 316), and 2-(2-hydroxyethyl-tridecyl-amino)ethanol (MW 288) [23]. These chemicals may possess anticancer effects with either each one or their combination.

S. horneri active component has been shown to possess a stimulatory effect on osteoblastic bone formation and an inhibitory effect on osteoclastic bone resorption in vitro, thereby increasing bone mass [4-7]. Intake of S. horneri extract caused preventive effects on bone loss in osteoporosis animal models and healthy human [8-10]. Functional food factor S. horneri extract may be usefulness as an osteogenic factor in preventing osteoporosis in human subjects. Moreover, S. horneri active component is speculated to possess suppressive effects on bone loss induced by breast cancer cell bone metastasis. Intake of S. horneri active component may be a useful tool in the prevention and therapy in breast cancer bone metastasis. This remains to be elucidated.

In conclusion, marine algae S. horneri active component was found to suppress proliferation and stimulate apoptotic cell death in human breast cancer MDA-MB-231 bone metastatic cells in vitro. Thus, S. horneri active component was demonstrated to possess anticancer effects in human breast cancer cells in vitro.

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Author contribution

The author (M.Y.) contributed to the design and conduct of the study, collection, analysis, and interpretation of data, and manuscript writing. Other authors (T.M.) partly contributed to conduct of the study. All authors have no conflicts of interest.

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