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Short communication

Induction of apoptosis in human cervical carcinoma HeLa cells by neoechinulin A from marine-derived fungus *Microsporum* sp.

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ABSTRACT

Recently, the relationship between apoptosis and cancer has been emphasized and the induction of apoptosis is recognized as one of the key mechanisms of anti-cancer agents. Marine-derived fungi are valuable sources of structurally diverse bioactive compounds with anticancer activity. In the present study, a marine-derived fungus, *Microsporum* sp. was cultured and a prenylated indole alkaloid, neoechinulin A was isolated from the culture broth extract. Neoechinulin A has shown cytotoxic effect on human cervical carcinoma HeLa cells and its apoptosis induction in HeLa cells was investigated by the expressions of p53, p21, Bax, Bcl-2, Caspase 9, and Caspase 3 proteins. Western blot analysis has revealed that neoechinulin A could induce cell apoptosis through down-regulating of Bcl-2 expression, up-regulating of Bax expression, and activating the caspase-3 pathway. Collectively, these results suggest that neoechinulin A could be a potential candidate in the field of anticancer drug discovery against human cervical cancer.

1. Introduction

Cancer is a leading cause of death worldwide and a diverse group of diseases characterized by the uncontrolled proliferation of anaplastic cells which tend to invade surrounding tissues and metastasize to other tissues and organs [1]. Hence, development of new anticancer drugs represents today one of the most important research areas. An analysis of the number of chemotherapeutic agents and their sources indicates that over 74.8% of the approved drugs are derived from natural compounds [2]. Chemoprevention entails the use of specific naturally occurring dietary or synthetic agents to thwart cancer development and progression [3]. Nature-derived pharmaceuticals including marine-derived bioactives have drawn a great deal of attention from both the scientific community and the general public owing to their demonstrated ability to suppress cancers. Many potent natural products which display effective anticancer activities have been discovered in marine environment. Indeed, since the early 1990s, there has been a dramatic increase in the number of preclinical anticancer lead compounds from marine sources that have entered into human clinical trials [4].

Marine-derived fungi are a rich source of structurally diverse bioactive secondary metabolites with unprecedented skeletons and have shown various health beneficial biological activities [5,6]. A number of metabolites from marine-derived fungi possess antioxidant, antimicrobial, antityrosinase or skin whitening, cytotoxic or antitumour, quinone reductase induction, and antiplasmodial activities. Up to date, a lot of structurally and pharmacologically novel and interesting bioactive secondary metabolites have been isolated from marine-derived fungi. Secondary metabolites that are produced by the marine organisms including marine-derived fungi have more novel and unique structures due to the complex living organism and diversity of species and the bioactivities are much stronger than terrestrial organisms. Due to the unique ecological environment of the marine-derived fungi, it has been approved that the marine-derived fungi may produce novel chemical structures and diverse biological activities. This study focuses on extraction and isolation of bioactive secondary metabolite, neoechinulin A from marine-derived fungus *Microsporum* sp. and presents its potential apoptosis induction effect in human cervical carcinoma HeLa cells.

2. Materials and methods

2.1. Materials

Dimethylsulfoxide (DMSO), 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT), agarose and fetal bovine

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serum (FBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents and organic solvents such as methanol (MeOH), n-hexane, chloroform ($CHCl_3$), and ethyl acetate (EtOAc) were of the highest grade available commercially. Dulbecco's modified eagle medium (DMEM), penicillin/streptomycin, and the other materials required for culturing of cells were purchased from Gibco BRL, Life Technologies (Grand Island, NY, USA).

2.2. Isolation of neoechinulin A from marine-derived fungus *Microsporum sp.*

The fungal strain (MFS-YL) was isolated from the surface of a marine red alga *Lomentaria catenata*, collected at Guryongpo, Nam-Gu, PoHang in Republic of Korea in 2009. The fungal strain was cultured in YPG medium (0.5% yeast extract, 0.5% peptone, 1% glucose, and 60% seawater) and preserved in 10% glycerol with the YPG medium. The further culture was preceded with the same medium (YPG, 1 L × 20 Erlenmeyer flasks) to isolate secondary metabolites. The medium was sterilized (121 °C, 15 lb/in.^{2}) before the culture of fungal strain and inoculation was done after cooling down the media under the laminar flow. The fungus was cultured for 30 days at 25 °C and identified as *Microsporum* sp. based on the cellular fatty acid composition with a similarity index of 0.62 by the Korean Culture Center of Microorganisms, Seoul, Republic of Korea. The culture broth was separated from the mycelium by filtering through a filter paper (No. 2, Wattmann) and the pooled broth was extracted with ethyl acetate (1:1.5, v/v, broth:EtOAc). The broth extract was fractionated by silica gel flash chromatography (n-hexane:EtOAc and $CHCl_3$:MeOH) to yield ten fractions (Fr.1–Fr.10). Further purification was carried out by ODS column chromatography (H_2O:MeOH), followed by High Performance Liquid Chromatography (HPLC) (YMC ODS-A, MeOH).

2.3. Cytotoxicity assay and cell morphology changes

Human cervical carcinoma (HeLa cells) (ATCC, Manassas, VA, USA) were cultured and maintained in Dulbecco's modification of eagle's medium (DMEM, GIBCO, New York, USA) containing 100 μg/mL penicillin–streptomycin, 10% μg/mL fetal bovine serum (FBS) and maintained at 37 °C under a humidified atmosphere with 5% CO_2. Cytotoxicity levels of the isolated neoechinulin A from the marine-derived fungi, *Microsporum* sp. on HeLa cells were measured using 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method as described by Hansen et al. [7] with slight modifications. Cell lines were cultured in 96-well plates at a density of 5×10^3 cells/well. After 24 h, cells were washed with fresh medium and were treated with different concentrations of neoechinulin A. After the next 24 h of incubation, cells were visualized and photographed under fluorescence microscope (Leica, Wetzler, Germany) and then, cells were washed two times with phosphate buffered saline (PBS) and 100 μL of MTT solution (1 mg/mL) was added to each well. After next 4 h of incubation, MTT solution in each well was removed by suction and then 100 μL of dimethyl sulfoxide (DMSO) was added to solubilize the formazan salt. The optical density was measured at 540 nm by using UV microplate reader (Tecan Austria GmbH, Groedig, Austria). Relative cell viability was calculated compared to the non-treated blank group.

2.4. Detection of p53, p21, Bax, Bcl-2, Caspase 9, and Caspase 3 expressions through Western blot analysis

Western blotting was performed according to the standard procedures. Briefly, HeLa cells were cultured in DMEM at a density of 2×10^4 cells per 10 cm^2 cell culture dishes and incubated for 24 h at 37 °C under a humidified atmosphere with 5% CO_2. After that, cells were treated with different concentrations of neoechinulin A and incubated for 24 h. Then, cells were lysed with RIPA buffer containing 50 mM Tris–HCl (pH 8.0), 0.4% Nonidet P-40, 120 mM NaCl, 1.5 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 80 μg/mL leupeptin, 3 mM NaF and 1 mM DTT at 4 °C for 20 min. Then the cell lysate was centrifuged at 5000 *g* for 10 min to remove insoluble materials. The protein content in the supernatants was determined using BCA protein assay kit (Thermo Science, Rockford, USA). Same amount of protein in cell lysate supernatants were analyzed on 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech., UK), blocked with 5% skim milk, and hybridized with primary antibodies (diluted with 1:1000). After incubation with horseradish-peroxidase-conjugated secondary antibody at room temperature, immunoreactive proteins were detected using a chemo-luminescent ECL assay kit (Amersham Pharmacia Biotech., UK), according to the manufacturers instructions. Western blot bands were visualized using a LAS3000 luminescent image analyzer (Fujifilm Life Sciences, Tokyo, Japan).

2.5. Statistical analysis

Data were expressed as mean ± standard deviation of three independent determinations. The significance of differences between two samples was analyzed using the Student *t*-test. A *P*-value of <0.05 was taken as the level of statistical significance.

3. Results and discussion

3.1. Structure of neoechinulin A

Marine-derived fungi grow in unique and extreme habitats; they may have the capability to produce bioactive secondary metabolites. In the course of screening for new bioactive metabolites with potential use in pharmacology, we investigated a fungal strain isolated from surface of a marine red alga. This marine-derived fungus was identified as *Microsporum* sp. based on the gas chromatographic analysis of cellular fatty acid profile with a similarity index of 0.62 by the Korean Culture Center of Microorganisms, Seoul, Republic of Korea. Analysis of cellular fatty acid profile is now used routinely to characterize, differentiate, and identify genera of marine-derived fungi. Fungi produce fewer different fatty acids than bacteria do. The isolated fungal strain was cultured (20 L) for 30 days at 25 °C pH 7.6 in YPG medium. The culture broth and mycelium were separated and the filtered broth was extracted with ethyl acetate and dried under vacuum to get the broth extract (MFS-YL B, 1710 mg). The broth extract was fractionated by silica gel flash chromatography [(n-hexane:EtOAc = 10:1, 7:1, 5:1, 3:1, 1:1, and EtOAc 100%) and (EtOAc:MeOH = 1:1), from both 3 L] and obtained ten fractions (Fr.1–Fr.10). Among ten fractions, Fr.5 (118.33 mg) was further purified by ODS column with H_2O:MeOH = H_2O 100%, 9:1, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:5, 1:3, and MeOH 100%). Final purification of the Fr.5 by ODS column chromatography (H_2O:MeOH, 1:5) followed by HPLC (YMC ODS-A, MeOH) yielded neoechinulin A (34.3 mg) (Fig. 1). The chemical structure of the isolated neoechinulin A from broth extract of the marine-derived fungus, *Microsporum* sp. was determined according to the spectroscopic data and literature findings (Fig. 2).

Neoechinulin A (34.3 mg): colorless solid; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.41 (1H, d, H-4), 7.23 (1H, d, H-6), 7.11 (1H, s, H-8), 7.06 (1H, m, H-7), 6.12 (1H, dd, H-16), 5.11 (2H, m, H-17), 4.23 (1H, dd, J = 6.8 Hz, H-12), 1.53 (6H, m, J = 6.8 Hz, H-15a), 1.52 (3H, m, J = 1.36 Hz, H-12a) ^{13}C NMR (CD_3OD, 100 MHz) δ 168.9 (C-13), 162.4 (C-10), 146.3 (C-16), 146.1 (C-2), 136.9 (C-7a), 127.4 (C-3a), 124.8 (C-9), 122.7 (C-5), 121.3 (C-7), 120.0 (C-6), 114.5 (C-4), 112.8 (C-8),