Glioblastoma-Specific Anticancer Activity of Pheophorbide a from the Edible Red Seaweed *Grateloupia elliptica*

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The chlorophyll-related compound pheophorbide a (Pa) was successively purified from an edible red seaweed, *Grateloupia elliptica*, using silica, octadecyl silica column chromatography and reversed phase-high-performance liquid chromatography, as well as the cell cycle inhibitory and apoptotic effects of Pa being investigated in U87MG glioblastoma cells. The Pa exhibited strong anticancer effects in the absence of direct photo-irradiation against various cancer cell lines, including U87MG, SK-OV-3, and HeLa cells. Among the cancer cells, the strongest anticancer activity of Pa exhibited on U87MG cells with IC₅₀ values of 2.8 µg/ml. In addition, Pa specifically had cytostatic activity on glioblastoma cells rather than human umbilical vein endothelial cells. Analysis of the cell cycle distribution showed that Pa induced G0/G1 arrest of U87 MG cells. In addition, arrested cells induced late apoptosis and DNA degradation under dark condition. These results suggest that Pa isolated from *G. elliptica* is a potential glioblastoma-specific anticancer agent without side effects on normal cells.

**Keywords:** Glioblastoma, selective toxicity, pheophorbide a, anticancer, *Grateloupia elliptica*, cytostatic

Introduction

Glioblastoma multiforme (GBM) is the most common type of brain tumors and is a rapidly progressive and biologically aggressive disease with a high mortality rate, with patients usually surviving less than 12 months following initial diagnosis [29, 36]. GBM patients are normally treated with surgery followed by radiation and chemotherapy [1, 34]. However, several anti-GBM agents used as chemotherapeutics have displayed serious side effects in patients, owing to nonspecific cytotoxic effects on both tumor and normal cells [14]. Therefore, the development of safe GBM therapeutic agents without side effects has become an urgent issue, and many studies have been conducted to identify cytotoxic effects selective against only cancer cells [23, 31]. Recently, cell cycle inhibition and/or induction of apoptosis in glioblastoma cells have come to be appreciated as targets for the management of GBM [11]. Regulation of the cell cycle machinery may induce arrest in different phases of the cell cycle and thereby reduce the growth and proliferation of cancer cells [12]. Additionally, cell cycle arrest of cancer cells may be related to apoptosis, as well as the magnitude of DNA damage [6].

Natural products derived from marine resources are attractive sources for the development of new medicinal and therapeutic agents, and more than 3,000 new anticancer compounds have been identified from marine organisms, such as tunicates, microorganisms, sponges, and sea hares [28, 30]. In a recent study, marine seaweed-derived natural anticancer compounds were isolated from brown algae, such as fucoxanthin from *Ishige okamurae* [20] and eckol from *Ecklonia cava* [16], as well as red algae, including elatol from *Laurencia microcladia* [10]. However,
The activity of Pa was investigated.

Analytical data, and the glioblastoma-specific anticancer agents, we observed that methanol extracts of Grateloupia elliptica effectively inhibited U87MG glioblastoma cells. The red seaweed G. elliptica is one of the most popular edible seaweeds in Northeast Asian countries, where it is also commonly used for making glue. Although the potent α-glucosidase inhibitory activity of bromophenols from G. elliptica has been previously established [21], the isolation of pheophorbide a (Pa) from G. elliptica, and its selective anticancer activity on glioblastoma cells in the absence of direct photo-irradiation, have not been reported. Therefore, in this study, we isolated Pa from G. elliptica, verified by the results of nuclear magnetic resonance (NMR) and other analytical data, and the glioblastoma-specific anticancer activity of the Pa was investigated.

Materials and Methods

Sample and Materials

The edible red seaweed G. elliptica was collected at a nearby seashore area in Jangho, Samcheok, Korea in March 2010. The raw sample was immediately frozen and stored at −70°C until further use.

Phosphate-buffered saline (PBS), trypsin, L-glutamine, fetal bovine serum, and Trizol reagent were purchased from Gibco-Invitrogen Co. (Paisley, UK). RPMI-1640 medium was purchased from Lonza Inc. (Walkersville, MD, USA). Agarose, propidium iodide, and 100-base-pair DNA ladder were obtained from Promega (Madison, WI, USA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Reversed-phase gel (ODS-AQ, YMC, Japan) and silica gel 60 (Merck, Germany) were used for column chromatography. All other chemicals and reagents used in this work were of analytical grade.

Purification and Identification of Pa from G. elliptica

The raw samples (G. elliptica, 10 kg) were extracted twice with methanol (20 L) at room temperature for 24 h. After filtration, the crude extracts were evaporated to dryness under a rotary evaporator and a vacuum drier at 30°C. The crude extracts (150.3 g) were then dissolved in distilled water and partitioned sequentially in three different solvents, n-hexane (HX), chloroform (CF), and ethyl acetate (EA), to fractionate polar and nonpolar compounds. The EA fraction (7.82 g) was then further fractionated using a silica gel column (Kieselgel 60, 70–230 mesh; Merck) with the following solvent conditions: HX/EAN (2:1, 200 ml) and HX/EAN/MeOH (2:1:0.2, 600 ml). The strongly active fractions (750 mg) were collected and further purified with C18 reversed-phase column chromatography (ODS-AQ, YMC, Japan) by step-gradient elution with each of the different percentages of methanol (80–100%, 100 ml). In addition, the active compound (57.2 mg) was purified by reversed-phase high-performance liquid chromatography (RP-HPLC) using a Waters HPLC system (breeze 1525; NY, USA) equipped with a Waters 2489 UV/Vis detector and a C18 analytical HPLC column (Chromolith performance RP-18e; Merck) at eluting conditions with a flow rate of 1 ml/min, an eluent of 90% acetonitrile, and a monitoring wavelength of 220 nm. Finally, the active compound (10.5 mg) was identified by comparing its 1H and 13C NMR data with the literature [17].

Cell Culture

Human glioblastoma cells (U87MG), mouse melanoma cells (B16-BL6), human epithelial carcinoma cells (HeLa), human cervical cancer cells (SiHa), and human ovarian cancer cells (SK-OV-3) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cancer cell lines were cultured in DMEM with 10% fetal bovine serum, 100 µg/ml streptomycin, 100 U/ml penicillin, and 3.7 mg/ml NaHCO3. Human umbilical vein endothelial cells (HUVEC) were obtained from human umbilical cord veins, essentially as described by Jaffe et al. [18]. The HUVEC were cultured in M199 with EGM-2 medium containing growth factor (Cambrex, Walkersville, MD, USA) supplemented with 100 µg/ml streptomycin, 100 U/ml penicillin, and 3.7 mg/ml NaHCO3. The various cancer cells and HUVEC were incubated in an atmosphere of 5% CO2 at 37°C and were subcultured every 3 days.

Anticancer Activity Assays

The anticancer activity of samples on various cancer cells was determined by MTT assay. Cancer cell lines (5 × 104 cells/well) were inoculated into a 96-well plate in triplicate, incubated overnight at 37°C in the presence of 5% CO2, treated with Pa, and further incubated for 48 h. Twenty microliter of MTT solution (5 mg/ml) was added to each well, and the plates were incubated at 37°C for 4 h. The supernatant was aspirated, and the MTT-formazan crystals generated metabolically viable cells were dissolved in 200 µl of DMSO. The absorbance at 570 nm was determined using a microplate reader (SpectraMax250; Molecular Devices, CA, USA). For the various cancer cell lines, 50% suppression of viability was calculated relative to the control (blank) treatment and expressed as IC50.

The cytotoxic effect of Pa was determined using a viability assay of confluent cells (2 × 105 cells/well) as a resting cell after U87MG cells were treated with 50 µg/ml of Pa during 48 h under dark condition. In addition, the cytostatic effect of Pa was determined by cell growth assay at the same condition to the above cytotoxic assay except for the initial cell concentration (5 × 105 cells/well).