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Brown algae phlorotannins enhance the tumoricidal effect of cisplatin and ameliorate cisplatin nephrotoxicity

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HIGHLIGHTS

- Phlorotannin-rich extract of *Ecklonia cava* (PREC) enhanced the tumor growth-inhibitory effect of cisplatin and ameliorated cisplatin-induced nephrotoxicity *in vivo*.
- PREC enhanced ovarian cancer cell apoptosis by cisplatin via the ROS/Akt/NFκB pathway and suppressed cisplatin-induced normal kidney cell damage.
- Dieckol, a major phlorotannin of PREC, significantly enhanced the tumoricidal effect of cisplatin *in vivo*.

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ABSTRACT

Objective. The clinical application of cisplatin is limited due to its drug resistance and side effects. We investigated the effect of a phlorotannin-rich extract from the edible brown alga *Ecklonia cava* (PREC) and its major phlorotannin (dieckol) on cisplatin responsiveness and side effects.

Methods. The A2780 and SKOV3 ovarian cancer cell lines and the SKOV3-bearing mouse model were used. The MTT assay was applied to assess cell viability, and the annexin V assay was employed for apoptosis analysis. Reactive oxygen species (ROS) production and protein expression were assessed by H_2DCFDA staining and Western blotting, respectively.

Results. We found that PREC enhanced the tumor growth-inhibitory effect of cisplatin and diminished cisplatin-induced nephrotoxicity and weight loss in SKOV3-bearing mice. PREC augmented cisplatin-induced apoptosis by activating caspases in SKOV3 and A2780 ovarian cancer cells. In addition, a combination of PREC and cisplatin-induced ovarian cancer cell apoptosis by downregulating the Akt and NFκB pathways. We further demonstrated that PREC increased intracellular ROS and that antioxidants significantly attenuated Akt-NFκB activation and apoptosis in ovarian cancer cells. In contrast, PREC inhibited cisplatin-induced ROS production and cell death in normal HEK293 kidney cells. Dieckol, a major compound in PREC, significantly enhanced the inhibition of tumor growth by cisplatin with less weight loss and kidney damage in a mouse model.

Conclusion. These data suggest that brown algae phlorotannins may improve the efficacy of platinum drugs for ovarian cancer by enhancing cancer cell apoptosis via the ROS/Akt/NFκB pathway and reduce nephrotoxicity by protecting against normal kidney cell damage.

Introduction

Ovarian cancer is the most lethal gynecological malignancy, as most patients with ovarian cancer are diagnosed at late stages due to a lack of effective screening strategies and specific symptoms associated with early stage disease [1]. Despite the great achievements made over the past three decades in cytoreductive surgery and combination chemotherapy, ovarian cancer remains a significant threat to women internationally [2].

Cisplatin, or *cis*-diamminedichloroplatinum, has been used to treat a variety of malignancies including ovarian cancer. However, the administration of cisplatin is often associated with serious side effects including nephrotoxic and neurotoxic events. The accompanying toxicity to normal tissue and the acquisition of drug resistance poses major problems during cisplatin therapy [3,4]. The combined use of

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two or more chemotherapy agents is often advantageous, as it may permit lowering drug dosages and consequently decreasing cytotoxicity, which reduces the opportunity for the development of drug resistance by cancer cells [5]. Therefore, identifying agents that can sensitize tumor cells to cisplatin with no or less toxicity to normal tissue would have an important impact on cisplatin-based therapy.

Many studies have demonstrated the anticancer effect of natural materials from terrestrial and marine resources. Marine-derived natural products are known for their huge diversity of chemical structures, and their unique structures are frequently associated with special mechanisms of action by which they may elicit unexpected biological activities [6]. Marine algae are good candidates for these unique biological activities due to their diverse classes of active secondary metabolites. Polyphenols are one of the most common classes of secondary metabolites in marine plants. The unique polyphenolic compounds phlorotannins, which are formed by the polymerization of phloroglucinol (1,3,5-tryhydroxybenzene) units, have been identified in *Ecklonia* sp. [7]. Phlorotannins such as eckol (a closed-chain trimer of phloroglucinol), 6,6′-bieckol (a hexamer), dieckol (a hexamer), and phlorofucofuroeckol (a pentamer) have been identified in the edible brown algae *Ecklonia cava* (*E. cava*) and have been extensively studied for their potential health benefits. For example, the promising effects of *E. cava* against radical-mediated oxidative stress [8], photodamage [9], allergy [10], diabetes [11], inflammation [12], and viral and microbial infections [13] have been reported. Phlorotannins are believed to be promising as nutraceuticals and pharmaceuticals due to their vast range of biological activities. Thus, a better understanding of the molecular mechanisms behind the modulation of cellular responses is needed to develop and optimize new therapeutic strategies using *E. cava*.

In the present study, we investigate the potential effect of the phlorotannin-rich extract of the edible brown alga *E. cava* (PREC) to enhance cisplatin cytotoxicity and reduce kidney damage in ovarian cancer *in vivo* and *in vitro*. We also examined the reactive oxygen species (ROS)-mediated mechanisms of action of the combination of cisplatin and PREC. In addition, we studied the role of dieckol, a major phlorotannin in PREC, for PREC-induced anticancer effects.

Materials and methods

Materials

PREC and dieckol (>99% purity) used for this study was kindly supplied by Livechem, Inc. (Daejeon, South Korea). The method to prepare the phlorotannins-rich extract of *E. cava* (PREC) has been previously described [14]. The total polyphenol content of PREC as phloroglucinol equivalent was about 98%, and major phlorotannins in the PREC were as follows: 2-*O*-(2,4,6-trihydroxyphenyl)-6,6′-bieckol, 6,6′-bieckol, 8,8′-bieckol, 7-phloroeckol, eckol, phlorofurofukoeckol, and dieckol (16.6%), as determined by HPLC [Waters, column:Spherisorb S_{10}ODS2 column (20 × 250 mm^2); eluent: 30% aqueous EtOH; flow rate: 3.5 ml/min]. RPMI 1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies Inc. (NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), RNase A, leupeptin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), Triton X-100, and propidium iodide (PI) were purchased from Sigma Chemical Co. (MO, USA). Antibodies against p65, p50, Bcl_2, BclxL, FLIP/L, Akt, proliferating cell nuclear antigen (PCNA), and β-actin were purchased from Santa Cruz Biotechnology (CA, USA). Antibodies against X-linked inhibitor of apoptosis protein (XIAP), caspase-3, and caspase-8 were purchased from BD Biosciences, Pharmingen (CA, USA). Antibodies against caspase-9 and phospho-Akt were purchased from Cell Signaling Technology (MA, USA). pcDNA3-Akt-myr, pcDNA3-Akt-DN, and pCMV4-p65 were obtained from Addgene (MA, USA). z-VAD-fmk, z-DEVD-fmk, and z-LEHD-fmk were obtained from Calbiochem (CA, USA).

Cell cultures and MTT assay

Ovarian cancer SKOV3 and A2780 cells were obtained from American Type Culture Collection. Cells were cultured in RPMI 1640 supplemented with 5% fetal bovine serum, penicillin (100 U/ml) and streptomycin sulfate (100 μg/ml) (Life Technologies, NY, USA) in a humidified atmosphere of 5% CO_2 95% air at 37 °C. Cell viability was assessed using an MTT assay. Briefly, the cells (5×10^4) were seeded in each well containing 50 μl of RPMI medium in a 96-well plate. After 24 h, cisplatin and/or PREC were added. After 48 h, 50 μl of MTT solution (5 mg/ml in PBS) was added to the medium, and the cells were incubated at 37 °C for 4 h. The optical density was measured at 540 nm using a microplate spectrophotometer (SpectraMax; Molecular Devices, CA, USA) to determine the cell viability.

Propidium iodide (PI) staining and annexin V and PI double staining

Cells were fixed and permeabilized with 70% ice-cold ethanol at 4 °C for 1 h. Cells were washed once with PBS and resuspended in a staining solution containing PI (50 μg/ml) and RNase A (250 μg/ml). The mixture was incubated for 30 min at room temperature in a dark place and analyzed by FC500 fluorescence-activated cell sorting (FACS) cater-plus flow cytometry (Beckman Coulter, CA, USA). A total of 10,000 events were acquired for analysis using Cell Quest software. For annexin V and PI double staining, cells were suspended with 100 μl of binding buffer and stained with 5 μl of FITC-conjugated annexin V and 5 μl of PI (50 μg/ml). The mixture was incubated for 15 min at room temperature in a dark place and analyzed by FACS.

Western blot analysis

Protein samples of cell lysate were mixed with an equal volume of 5× SDS sample buffer, boiled for 4 min, and then separated on 10–12% SDS–PAGE gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes. The membranes were blocked in 5% non-fat dry milk for 1 h, washed, and incubated with specific antibodies in Tris-buffered saline (TBS) containing Tween-20 (0.1%) overnight at 4 °C. Primary antibodies were removed by washing the membranes three times in TBS-T, and then the membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary antibody for 1 h, and visualized using the ECL chemiluminescent system (Amersham Pharmacia Biotech, ON, Canada). Following three washes in TBS-T, immuno-positive bands were visualized by enhanced chemiluminescence and exposed to Image Quant LAS-4000 (Fujifilm Life. Sciences, Tokyo, Japan).

Measurement of reactive oxygen species (ROS)

The intracellular accumulation of ROS was determined using the fluorescent probe H_2DCFDA. H_2DCFDA was commonly used to measure H_2O_2. Cells were collected by centrifugation 30 min before treatment with the cytotoxic agents, resuspended in PBS, and loaded with 20 μM H_2DCFDA. The fluorescence was measured at the desired time intervals by flow cytometry.

Animal study

BALB/c athymic female nude mice ($n = 20$) weighing 20–25 g from NARA Biotech (Seoul, Korea) were used for the studies. Ovarian carcinoma was created by subcutaneously (s.c.) inoculating 5×10^6 SKOV3 cells (100 μl) into the flank of each mouse and tumors were allowed to grow for 1 week. The tumor-bearing mice were randomly divided into 5 or 6 groups (5 mice/group). For 4 consecutive weeks, PREC (75 and 150 mg/kg) or dieckol (50 and 100 mg/kg) was given orally three times per week while cisplatin was administered via intraperitoneal (i.p.) injection at doses of 3 mg/kg/day three times per week. The