Phytomedicine 39 (2018) 25–32

Contents lists available at ScienceDirect

Phytomedicine

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

Cyclocommunol induces apoptosis in human oral squamous cell carcinoma partially through a Mcl-1-dependent mechanism

Jing-Ru Weng[a,*], Li-Yuan Bai[b,c,1], Horng-Huey Ko[d,1], Yi-Tung Tsai[e]

[a] Department of Marine Biotechnology and Resources, National Sun Yat-sen University, Kaohsiung 80424, Taiwan
[b] College of Medicine, China Medical University, Taichung 40402, Taiwan
[c] Division of Hematology and Oncology, Department of Internal Medicine, China Medical University Hospital, Taichung 40447, Taiwan
[d] Department of Fragrance and Cosmetic Science, College of Pharmacy, Kaohsiung Medical University, Kaohsiung 80715, Taiwan
[e] Department of Biological Science and Technology, China Medical University, Taichung 40402, Taiwan

ARTICLE INFO

Keywords:
Cyclocommunol
Breadfruit
Oral cancer
Autophagy
Apoptosis
Mcl-1

ABSTRACT

Background: Crude extract of breadfruit has been reported to have antitumor activity against various cancer cell lines with unknown mechanism.
Purpose: This study aims to investigate the proapoptotic effect of cyclocommunol (CYC), a prenylflavonoid from breadfruit, in two oral squamous cell carcinoma (OSCC) cell lines, SCC2095 and Ca922.
Methods: The antiproliferative effects of CYC were assessed by MTT assays and PI/annexin V analysis. SCC2095 cells were transiently transfected with Mcl-1 plasmid in overexpression experiment. Other methods used to investigate the mechanism of CYC included Western blotting, acridine orange staining and confocal microscopic visualization.
Results: Our results showed that CYC suppressed the viability of SCC2095 and Ca922 with IC_{50} values at 48 h of 4.2 and 5.0 μM, respectively. This decrease in viability occurred in a caspase-dependent apoptotic manner. In addition, CYC down-regulated the phosphorylation/expression of Akt/mTOR and Mcl-1, accompanied by reactive oxygen species generation, and autophagy induction. Notably, overexpression of Mcl-1 using Mcl-1-tag-myc partially rescued CYC-mediated caspase-3 activation, PARP cleavage, and cytotoxicity. In summary, our study demonstrated the proapoptotic activity of CYC on OSCC, partially through down-regulation of Mcl-1.
Conclusion: CYC from breadfruit has translational value as a proapoptotic agent for OSCC.

Introduction

Oral squamous cell carcinoma (OSCC) represents 95% of all head and neck cancers, and has an annual incidence of 275,000 cases worldwide (Sinevici and O'Sullivan, 2016). Due to the incidence of OSCC is increasing, the cost of managing late stage OSCC's was up to twice as expensive as managing early stage (Reddy et al., 2010; Speight et al., 2006). For OSCC patients in USA and Germany, the estimated cost was €893 million and € 1.0 to 1.9 million per year, respectively (Lee et al., 2004). In addition, it has been reported that the patient-reported outcomes scores such as physical disability, pain, fatigue, and emotional health were decreased significantly from diagnosis through the treatment period and recovered in the first-year post-treatment between 2004 and 2014 (Wissinger et al., 2014). Surgical intervention with or without adjuvant radiotherapy is the standard treatment modality for OSCC in early stage. For locally advanced or metastatic OSCC, treatment modalities include radiotherapy, chemotherapy, molecular targeted therapy, and immunotherapy. However, recurrence and drug resistance are important factors that contribute to the high morbidity and mortality of OSCC, and highlight the urgency of developing new strategies for the treatment of OSCC.

In tropics, breadfruit (*Artocarpus altilis*) provides higher-quality proteins to prevent malnutrition and child death (Liu et al., 2015). In addition to being a source of food and energy, recent studies have demonstrated that crude extracts of breadfruit exhibit several pharmacological activities, such as reducing cadmium-induced renal toxicity (Adaramoye and Akanni, 2016a), preventing damage from ultraviolet radiation (Tiraravesit et al., 2015), decreasing cholesterol-induced hypercholesterolemia (Adaramoye and Akanni, 2014), lowering blood pressure (Nwokocha et al., 2012), exerting antimicrobial activity (Jalal et al., 2015), and suppressing tyrosinase activity (Lan et al., 2013). Furthermore, crude extracts of breadfruit have been

* Corresponding author at: 70 Lienhai Road, Kaohsiung 80424, Taiwan.
E-mail address: jrweng@mail.nsysu.edu.tw (J.-R. Weng).
[1] Li-Yuan Bai and Horng-Huey Ko have contributed equally to this work.

https://doi.org/10.1016/j.phymed.2017.12.004
Received 3 July 2017; Received in revised form 1 November 2017; Accepted 4 December 2017

demonstrated to inhibit the growth of various cancer cell lines, including pancreatic cancer and breast cancer (Arung et al., 2009; Nguyen et al., 2014). For example, the methanolic extract of breadfruit inhibited signal transducer and activator of transcription 3 (STAT3) activity as well as the phosphorylation of STAT3 but not STAT3 expression in prostate cancer cells (Jeon et al., 2015). Diethylether extract of wood from breadfruit increased the percentages of sub G1 cells in breast cancer cells (Arung et al., 2009).

Flavonoids, sterols, fatty acids, carbohydrates, and chalcones have been isolated from breadfruit (Golden and Williams, 2001; Lan et al., 2013). A previous report showed that Artocarpin, a prenylated flavonoid, induced apoptosis through modulating MAPK and Akt/mTOR pathways in cutaneous squamous cell carcinoma cells (Hu et al., 2015). Among them, flavonoids have been reported to have chemopreventive activity, and to reduce the risk of getting chronic metabolic disorders and cancer, including OSCC (Iriti and Varoni, 2013).

Cyclocommunol (CYC), a prenylflavonoid isolated from breadfruit, has been shown to have antityrosinase and antiplatelet activities (Lan et al., 2013; Lin et al., 1993). CYC was found to inhibit the growth of human hepatoma and gastric cancer cells with IC_{50} values between 16 and 80 μM in a screening study to identify the active antitumor constituent of extracts from breadfruit (Ma et al., 2010). In this study, we aimed to evaluate the proapoptotic effect of CYC on OSCC cells, and to investigate the relevant mechanism.

Materials and methods

Reagents, antibodies, and plasmids

Cyclocommunol (CYC) was isolated and characterized as described previously (Lan et al., 2013). Structure identification and purity of this compound (Fig. S1–S4) were identified by spectroscopic methods and comparison with the reported spectral data ((Liou et al., 1993). All agents (CYC and the other agents used as positive control including etoposide, rapamycin, glutathione, and *N*-acetylcysteine) were dissolved in DMSO, diluted in culture medium, and added to cells at a final DMSO concentration of 0.1%. Antibodies against the following biomarkers were obtained from Cell Signaling Technologies (Danvers, MA, USA): PARP, caspase-3, caspase-9, Akt, p-^{473}Ser Akt, Mcl-1, p-^{139}Ser H2AX, p-^{15}Ser p53, p53, p-^{2448}Ser mTOR, mTOR, Bax, survivin, p62, LC3B, Atg5, and β-actin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Mcl-1 plasmid was obtained from OriGene Technologies, Inc (Rockville, MD, USA). The GFP-LC3 plasmid was purchased from Addgene (Cambridge, MA, USA). Plasmids expressing vector (pCMV6-Entry) and Mcl-1 (Myc-DDK-tagged) were purchased from OriGene Technology (Rockville, MD, USA). The enhanced chemiluminescence system for detection of immunoblotted proteins was from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Other chemicals and reagents were obtained from Sigma-Aldrich unless otherwise noted.

Cell culture

SCC2095 human oral cancer cells were kindly provided by Professor Susan R. Mallery (The Ohio State University) and cultured in DMEM/F12 (Gibco, Grand Island, NY, USA). Ca922 cells were purchased from the Japanese Cancer Research Resource Bank and cultured in MEM (Gibco), respectively. All culture medium for cancer cells were supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY) and penicillin (100 U/ml)/streptomycin (100 μg/ml) (Invitrogen). All cell types were cultured at 37 °C in an atmosphere of 5% CO_2.

Cell viability analysis

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay in 6 replicates as described previously (Bai et al., 2016). Briefly, 5×10^3 cells/well were seeded in 96-well flat-bottomed plates; then, 24 h later, cells were treated with DMSO vehicle or drug at the indicated concentrations. After 24 h, the medium was removed, replaced by 200 μl DMEM/F12 containing 0.5 mg/ml of MTT and cells were incubated in the CO_2 incubator at 37 °C for 2 h. Supernatants were aspirated from the wells, and the reduced MTT dye was solubilized in 200 μl/well DMSO. Absorbance at 570 nm was determined using a plate reader.

Immunoblotting

Western blot analysis was performed as reported previously (Bai et al., 2016). Briefly, treated cells were washed with phosphate-buffered saline (PBS), resuspended in SDS sample buffer, sonicated for 5 s, and then boiled for 5 min. After brief centrifugation, equivalent amounts of proteins from the soluble fractions of cell lysates were resolved in 10% SDS-polyacrylamide gels on a Minigel apparatus, and transferred to a nitrocellulose membrane using a semidry transfer cell. The transblotted membranes were washed thrice with TBS containing 0.05% Tween 20 (TBST). After blocking with TBST containing 5% nonfat milk for 120 min, the membranes were incubated with the appropriate primary antibodies at 1:500 dilution (with the exception of anti–β-actin antibody, 1:2000) in TBST–5% low fat milk at 4 °C overnight, and then washed thrice with TBST. Membranes were probed with goat anti-rabbit or anti-mouse IgG-horseradish peroxidase conjugates (1:2500) for 90 min at room temperature, and washed thrice with TBST. The immunoblots were visualized by enhanced chemiluminescence.

Flow cytometry

Cells (2×10^5/3 ml) were treated with DMSO or CYC at the indicated concentrations for 24 h. After being washed twice with ice-cold PBS, cells were fixed in 70% cold ethanol for 4 h at 4 °C. For apoptosis evaluation, cells were stained with Annexin V and propidium iodide (PI) (1 μg/ml), counted on a BD FACSAria flow cytometer (Becton-Dickinson, Germany), and analyzed by ModFitLT V3.0 software program. In the inhibitor study, cells were pre-treated with chloroquine (CQ, 10 μM) or 3-methyladenine (3-MA, 20 μM) for 15 min, followed by incubation with DMSO or CYC. ROS production was detected using the fluorescence probe 2′,7′-dichlorodihydrofluoresceindiacetate (H2DCFDA; Molecular Probes, Eugene, OR, USA) according to the manufacturer's instruction. Briefly, cells were treated with DMSO or CYC with or without pre-treatment of *N*-acetylcysteine (NAC) or glutathione (GSH) for 15 min. After 3 h, the cells were washed with PBS twice and then stained with H2DCFDA (5 μM) at 37 °C for 30 min. After washing with PBS, fluorescence intensity for ROS generation was assessed using a flow cytometer.

Detection and quantification of autophagosomes with acridine orange staining by flow cytometry

The volume of the cellular acidic compartment was visualized by acridine orange staining (Paglin et al., 2001; Wilson et al., 2011). Cells were treated with DMSO or CYC or rapamycin at the indicated concentration for 48 h, then stained with acridine orange (1 mg/ml) for 15 min. After removing the acridine orange, cells were washed with once with PBS, and suspended in 0.5 ml of PBS. For quantification of the number of cells with acidic vesicular organelles (AVO), a flow cytometer (BD FACSCanto II) with red (650 nm, stained by cytoplasmic vesicles) *vs* green (510–530 nm, stained nuclei) fluorescence (FL3/FL1) from cells illuminated with blue (488 nm) excitation light were measured. A minimum of 10,000 cells within the gated region were analyzed. The fluorescence intensity is proportion to both the degree of acidity and the volume of the cellular acidic compartment.