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Mechanism of sappanchalcone-induced growth inhibition and apoptosis in human oral cancer cells

Young-Man Lee [a], Youn-Chul Kim [c], Byeong-Jun Choi [b], Deok-Won Lee [b], Jung-Hoon Yoon [d,1,*], Eun-Cheol Kim [a,1,*]

[a] Department of Maxillofacial Tissue Regeneration, School of Dentistry and Institute of Oral Biology, Kyung Hee University, Seoul, Republic of Korea
[b] Department of Oral and Maxillofacial Surgery, School of Dentistry, Kyung Hee University, Seoul, Republic of Korea
[c] College of Pharmacy, Wonkwang University, Iksan, South Korea
[d] Department of Pathology, School of Dentistry, Chosun University, Kwangju, South Korea

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ABSTRACT

Sappanchalcone, a flavonoid extracted from *Caesalpinia sappan*, exhibits cytoprotective activity, but the molecular basis for the anticancer effect of sappanchalcone has not been reported. In this study, we examined whether sappanchalcone could inhibit the growth of human primary and metastatic oral cancer cells, and we analyzed the signaling pathway underlying the apoptotic effects of the compound in this process using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assays, fluorescence microscopy, flow cytometry, and Western blotting. Sappanchalcone-treated oral cancer cells showed an increased cytosolic level of cytochrome *c*, downregulated Bcl-2 expression, upregulated Bax and p53 expression, caspase-3 and -9 activation, and poly (ADP-ribose) polymerase cleavage. Furthermore, sappanchalcone induced activation of p38, extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK), and Nuclear factor *k* B (NF-κB), as demonstrated by the phosphorylation of each mitogen-activated protein kinases (MAPKs), the degradation of inhibitor of NF-κα (IκB-α), increased expression of nuclear p65, and NF-κB-DNA binding. Inhibition of the expression of p38, ERK, JNK, and NF-κB by pharmacological inhibitors reversed sappanchalcone-induced growth inhibition and apoptosis. These results provide the first evidence that sappanchalcone suppresses oral cancer cell growth and induces apoptosis through the activation of p53-dependent mitochondrial, p38, ERK, JNK, and NF-κB signaling. Thus, it has potential as a chemotherapeutic agent for oral cancer.

1. Introduction

Oral squamous cell carcinoma (OSCC) is the most common cancer of the oral and maxillofacial region, with more than 300,000 new cases reported annually worldwide. Based on currently available clinical assessment and treatment methods, patients are often diagnosed at a late stage of the disease and the 5-year survival rate has remained relatively low (~50–60%) (Schliephake, 2003).

Surgical treatment for oral cancer can cause functional and aesthetic impairment, leading to withdrawal and social isolation (Hopper et al., 2004). Complications of radiotherapy can impair wound healing and further complicate surgical salvage after a failed procedure (Bodin et al., 2004). Conventional chemotherapeutic agents have been associated with numerous significant clinical complications, including nausea, hair loss, and pancytopenia; thus, alternative and less toxic chemical treatments for oral cancer are required (Yamachika et al., 2004). One approach for developing clinically effective chemotherapeutic agents is to screen traditional medicinal plants that have been used for thousands of years with few side effects for their anticancer activities (McCann, 1997). Previously, we demonstrated that highly purified sulfur (Lee et al., 2008) and herbal medicines such as *Coptidis rhizoma* and verticinone had antitumor effects on oral cancer cells in vitro (Lee et al., 2006; Yun et al., 2008).

Caesalpinia sappan (*C. sappan*) has been used in Oriental medicine as an antitumor agent, and extracts of *C. sappan* are reported to have several pharmacological activities, including anti-analgesic, anti-inflammatory, anti-hypercholesterolemic, and sedative effects (Saitoh et al., 1986; Nagai et al., 1986; Xie et al., 2000). In addition, we previously reported that a chloroform extract of *C. sappan* induced cell death and growth inhibition in oral cancer cells, which have increased levels of p53 and p21 (Kim et al.,

* Corresponding authors. Addresses: Department of Pathology, Chosun University, School of Dentistry, 375 Seoseok-dong, Dong-gu, Gwangju 501-759, Republic of Korea. Tel.: +82 62 230 6879; fax: +82 62 230 6880 (J.-H. Yoon), Department of Maxillofacial Tissue Regeneration, School of Dentistry, Kyung Hee University, 1 Heogi-dong, Dongdaemun-gu, Seoul 130-701, South Korea. Tel.: +82 2 961 0746; fax: +82 2 960 1457 (E.-C. Kim).
E-mail addresses: jhyoon@chosun.ac.kr (J.-H. Yoon), eckim@khu.ac.kr (E.-C. Kim).
[1] These authors contributed equally to this work.

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2005). Moreover, we showed that a single compound isolated from *C. sappan* heartwood, isoliquiritigenin 2′-methyl ether, had anti-oral cancer effects involving mitogen-activated protein kinases (MAPKs) and the Nuclear factor κ B (NF-B) pathway (Lee et al., 2010).

Sappanchalcone, a bioactive flavonoid isolated from *C. sappan*, has been demonstrated to exert many biological effects, including neuroprotective activities (Moon et al., 2010), anti-inflammatory effects (Washiyama et al., 2009), inhibitory effects on antigen-induced beta-hexosaminidase release (Yodsaoue et al., 2009), and anti-influenza virus activity (Liu et al., 2009). Furthermore, we showed that sappanchalcone had cytoprotective effects against glutamate-induced oxidative stress in hippocampal cells (Jeong et al., 2009). In addition, we demonstrated that sappanchalcone prevented reactive oxygen species-induced damage in human dental pulp cells and inhibited lipopolysaccharide (LPS)-induced inflammatory mediators in human periodontal ligament (PDL) cells (Jeong et al., 2010). However, the effects of sappanchalcone on cancer cells have not been reported. The aim of this study was to examine the chemotherapeutic effect of sappanchalcone on human primary and metastatic oral cancer cells in vitro.

2. Materials and methods

2.1. Reagents

Sappanchalcone was isolated from Heartwood of *Caesalpinia sappan* as we described previously (Jeong et al., 2009). Antibody against NF-kB p65, IkB, or phosphorylated isoforms of IkB, STAT3, ERK, JNK, and p38 was purchased from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco BRL (Grand Island, NY). All other chemicals were obtained from Sigma (St. Louis, MO), unless indicated otherwise.

2.2. Cell culture

The cell line HNSCC4 (HN4) from a primary $T_3N_0M_0$ carcinoma of the mouth floor, and cell line HNSCC12 (HN12), from a metastatic carcinoma of the oral cavity (Cardinali et al., 1995), were derived in the laboratory of Dr. John F. Ensley (Wayne State University). Cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were dissociated with 0.25% trypsin just before transferring for experiments and counted using a hemocytometer.

2.3. Antiproliferative assay

Cell viability was determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were seeded in flat-bottomed 96-well plates, at 1×10^5 cell/well 24 h. prior to treatment. The cells were treated for various lengths of time with the agents indicated. Then 25 μl of 5 mg/ml MTT was added to each well. After 4 h, incubation at 37 °C, 100 μl of lysing buffer was added. The buffer consisted of 20% w/v of sodium deodoxyl sulfate in 0.1% of HCl solution. The plates were incubated for a further analysis on an ELISA reader at 570 nm.

2.4. Flow cytometry

Propidium iodide (PI) staining cells were seeded at 5×10^5 cells/well in six-well plates. After 24 h, cells were treated with Sappanchalcone for the 3 days. After treatment, cells were harvested and pelleted by centrifugation (400 × *g*, 4 °C, 5 min). The cells were fixed with cold 75% ethanol for 24 h and then stained with PI solution, consisting of 45 mg/mL PI, 10 mg/mL RNase A, and 0.1% Triton X-100. After incubation in the dark at 4 °C for 1 h, fluorescence-activated cells were sorted using the FACScan flow cytometer, and the data were analyzed using Cellfit Analysis Software.

2.5. Fluorescein isothiocyanate (FITC)-annexin V and propidium iodide (PI) double staining

Cells (5×10^5) were seeded in 6-well plates, incubated for 24, and then treated with or without Sappanchalcone, and the incubation was continued for 3 days. After treatment, the cell pellet was prepared in a FACStar tube containing annexin V-FITC solution and incubated in 5% CO_2 at 37 °C. The PI solution (without NP-40) was then added, and the ratio of PI-positive and annexin V-positive cells was measured using the flow cytometer.

2.6. Morphological analysis of apoptosis by staining with 4′6-diamino-2-phenylindole dihydrochloride (DAPI)

To confirm that the nuclei underwent morphological changes, the cells were cultured in 60 mm dishes overnight and then washed then twice in DMEM. The cells were then treated with sappanchalcone and fixed in 4% paraformaldehyde, after which they were incubated in 1 μg/ml DAPI solution for 30 min in the dark. The cells were then examined using a fluorescence microscope (Zeiss, Oberköchen, Germany).

2.7. Western blot analysis

Western blot analysis was performed by lysing cells in 20 mM Tris–HCl buffer (pH 7.4) containing protease inhibitor mixture (0.1 mM phenylmethanesulfonyl fluoride, 5 mg/ml aprotinin, 5 mg/ml pepstatin A, and 1 mg/ml chymostatin). Protein concentration was determined using the Lowry protein assay kit (P5626; Sigma). An equal amount of protein for each sample was resolved using 7% or 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and then electrophoretically transferred onto a Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Bio-Rad, Heracules, CA). The membrane was blocked with 5% skim milk and sequentially incubated with primary antibody (Santa Cruz Biotechnology) and horseradish peroxidase-conjugated secondary antibody followed by ECL detection (Amersham Pharmacia Biotech, Piscataway, NJ).

2.8. Electrophoretic mobility shift assay (EMSA)

Cells (1×10^6) were grown in 100-mm culture dishes and incubated for various times with sappanchalcone. Nuclear extracts were prepared from the cells as previously described (Im et al., 1997). The strand oligonucleotides containing the NF-κB sequences (Promega, Madison, WI) were 5′-end labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase. The binding reactions were carried out in a buffer containing 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.5 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 10% glycerol, 0.05% NP-40, and 2 μg poly (dI-dC) on ice for 15 min. The assay mixture was incubated with the radiolabeled oligonucleotides for 30 min at room temperature. After 6 × dye solution (0.1% bromophenol and 30% glycerol) was added, the mixture was immediately loaded and electrophoresed on a nondenaturing 6% polyacrylamide gel in 0.25 × Tris/Borate/EDTA (TBE) for 2 h at 150 V. The gels were then dried in a vacuum drier at 80 °C for 1 h and autoradiographed on X-ray films.