Anti-cancer Activities of β-mangostin Against Oral Squamous Cell Carcinoma

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Abstract

Oral squamous cell carcinoma (OSCC) is one of malignant tumors with poor prognosis resulting in major morbidity and mortality. The actual curative treatment is usually chemotherapy with concurrent radiation, sometimes combined with surgery. Unfortunately, the strength of the drugs used in chemotherapy causes side effects that can bring discomfort and inconvenience. Herbal remedies have been used for thousands of years with very minimal side effects and clearly merit extended research for their ability to selectively kill cancer cells.

The genus of Garcinia is well known as a medicinal plant in Southeast Asia. β-mangostin, a xanthone from the pericarps of various species of Garcinia has been shown to exhibit anti-cancer activities in various human cancer cells. However, no attempt has been made to explore the potential benefits of this xantone for treatment and/or prevention of OSCC. Here, we report that β-mangostin exhibits anti-proliferative effect and induces apoptosis in human oral squamous cell carcinoma cell lines HSC-3 and Ca-922. MTT assay showed that β-mangostin markedly inhibited proliferation of HSC-3 and Ca-922 cells in a dose- and time-dependent manner.

The apoptosis induced by β-mangostin was clearly detected by flow cytometry in both cell lines and confirmed by caspase activity assays. Moreover, quantitative RT-PCR revealed that apoptotic activity by β-mangostin in HSC-3 and Ca-922 cells is associated with an up-regulation of caspase -8 and pro-apoptotic PUMA genes. These results identify β-mangostin as a potential therapeutic agent for human oral squamous cell carcinoma.

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Introduction

Oral squamous cell carcinoma (OSCC) is a highly relevant problem of global public health. It is located within the top 10 ranking incidence of cancers and despite the progress in research and therapy, survival has not improved significantly in the last years, representing a continuing challenge for biomedical science.[1] Its incidence is increasing because it is often poorly understood by society in general and frequently ignored in its early stages.[2] The 5-year survival rates across nation remain lower than 50 percent.[3] The main treatment of solid OSCC is either radiotherapy or radical surgery depend on the location and the size of the tumor, which is often combined with adjuvant chemotherapy which depend on the stage of development of the cancer. Chemotherapy is very effective in killing cancer cells, but it also affects normal cells as well. The strength of the drugs used in chemotherapy causes side effects that can bring discomfort and inconvenience.[4]

Over the recent years, there has been growing interest in naturally occurring phytochemical compounds with anti-cancer

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potential, because they are relatively non-toxic, inexpensive and available in an ingestive form.[5-7]

Among these compounds are xanthones. Xanthones such as α- and β-mangostin are mostly naturally available in fruits especially in the pericarp of tropical fruit mangosteen (*G. mangostana*). Accordingly, several studies have found that xanthones demonstrate anti-inflammatory, antioxidant, antitumor, anti-allergic, antibacterial, antifungal, and antiviral activities.[8,9]

Numerous studies have reported the anticancer activities of α-mangostin on various cancer cells as reviewed by Ibrahim et al 2017 [10,11] as well as oral cancer cells.[12,13] On the other hand, anti-cancer properties β-mangostin have received very minimal attention.[14,15] Our previous study reported that β-mangostin demonstrated stronger cytotoxicity than α-mangostin on cancer cells tested, including OSCC HSC-3 cell line.[16] However, its anticancer mechanism for treatment and/or prevention of OSCC remain to be explored. The present *in vitro* study was conducted to determine anti-cancer properties of β-mangostin on OSCC HSC-3, and Ca922 cell lines. The anti-cancer activities were determined by measuring the potency of the compounds in inducing cytotoxicity, apoptosis and expression of pro-apoptotic genes.

Materials and methods

Preparation of β -mangostin solution

β-mangostin (obtained as a pale yellow needle crystal)[16] was dissolved in absolute ethanol at a concentration of 1 mg/mL, aliquoted and stored at -20°C in micro-centrifuge tubes wrapped in aluminum foil to avoid dimer formation. This stock will be diluted as needed in DMEM to produce the concentrations to be tested.

Cell lines

Human oral squamous cell carcinoma HSC-3 and Ca922 cell lines were gifted by Prof. Dr. Masa-Aki Ikeda, Tokyo Medical and Dental University, Japan. The cells were grown in DMEM medium supplemented with 10 % fetal bovine serum (FBS) and 1 % of Penicillin-Streptomycin in a humidified incubator with 5 % CO_2 at 37 °C. All reagents for the cell culture were from Gibco, US.

Cytotoxicity Assay

The cytotoxic activity was measured by MTT assay as first described by Mosmann[17] with slight modifications suggested by Ichwan et al.[18] Exponentially growing cells were sub-cultured in 96-well plates at an initial density of 3 X 10^4/well, treated with defined concentrations of β-mangostin or 0.1% DMSO (vehicle control). After 24 and 48 h of incubation, the media were then carefully removed from the wells without disturbing the cell pellets. The cells were then incubated in 30 µL of MTT (Sigma-Aldrich, US) at concentration of 5 mg/mL in Phosphate Buffer Saline (PBS) (Gibco, US) for 4 h. The intracellular formazan complex was dissolved in 150 µL of DMSO and the absorbance was measured by a microplate reader. The experiments were performed in triplicate and the relative cell viability (%) was expressed as a percentage relative to the vehicle (DMSO) control cells.

Flow Cytometry Analysis

The flow cytometry was performed to analyze the cell cycle using propidium iodide (PI) staining method as described by Taher et al.[19] Briefly the cells were grown overnight to ~80% confluency in 10 cm dishes. The cells were exposed to either DMSO or β-mangostin using the IC_{50} concentrations obtained from the MTT assay. The cells were trypsinized after 24 h of incubation and then washed with cold PBS solution following by fixation using 1 ml of 75 % ethanol in PBS at 15 min. The ethanol was removed by centrifugation and the cells were washed with ice cold PBS. The cell pellets were then resuspended in PBS containing 50 µg/mL PI (Invitrogen), 0.2 mg/mL RNase A (Sigma-Aldrich, US) and 0.1% Triton X-100 (Fisher Scientific). After incubation at 37 °C for 30 min, the cells were analyzed by Guava EasyCyte™ HT flow cytometer (Merck Millipore, US The percentage of each cell cycle phase was analyzed using Guava-InCyte Software (Merck Millipore, US) for Mac.

Caspase 3/7 Activity Assay

The caspase 3/7 activity was determined by using the caspase-Glo 3/7 assay Kit (Promega, US) as per manufacturer's instructions. The cells were grown overnight in 96-well flat clear bottom white plates at density of 3 X 10^4/well. The cells were treated in the same manner as mentioned in the flow cytometry experiment. The assay was conducted according to the manufacture protocol by adding the caspase-Glo 3/7 assay reagent directly to the well at intervals after exposure to samples up to

24 h. The absorbance was read in a luminometer 30 min after addition of the reagent into the wells. The caspase activities in cells were measured and expressed as fold increase from the baseline controls.

RNA extraction, cDNA synthesis & Quantitative RT–PCR

Total RNA were prepared using the TRI Reagent (Sigma-Aldrich). Complementary DNAs were synthesized from 5 mg of total RNA using the Omniscript Reverse transcriptase (Qiagen) according to the manufacturer's instructions. Real-time PCR was performed using QuantiTect SYBR Green PCR Kit (Qiagen) as per the manufacturer's recommendations. Amplification was carried out in a LightCycler (Roche) with SYBR green detection and melt curve analysis. Oligonucleotide primers used have been described previously: *caspase*-3 and *-8* and *PUMA*.[20,21] The amount of cDNA present in any given sample was normalized to the amount of DNA of the housekeeping gene beta-actin.[22] Reaction mixtures contained 2μL cDNA, 2x QuantiTect SYBR Green RT-PCR Master Mix, 300 nM each of forward and reverse primer and diethyl Nuclease free water to a final volume of 15 μL. All samples were run in triplicate and the melting curves obtained after PCR amplification confirmed the specificity of the SYBR green assays.

Results

Briefly, we examined whether β-mangostin affected HSC-3 and Ca922 morphology and cell growth. Observation under a phase-contrast microscope, treatment of HSC-3 and Ca922 cells with concentrations of β-mangostin 0.5 μM resulted in noticeably alterations in cell morphology and an impaired ability of cells to become confluent, with a concomitant increase of floating dead cells (Figure 1). The effects of β-mangostin on the proliferation of HSC-3 and Ca922 cells were measured by the MTT assay.

The cells were treated with graded concentrations (0 - 2 μM) of β-mangostin for 12 and 24 h. β-mangostin was found to reduce the of the cell's viability in a dose- and time-dependent manner in both cell lines (Figure 2). The IC_{50} values of β-mangostin against the HSC-3 and Ca922 cells at 24 h were 0.62 μM and 0.44 μM respectively. Whereas at 48 hours, the IC_{50} values of β-mangostin were 0.27 μM and 0.21 μM respectively (Figure 2).

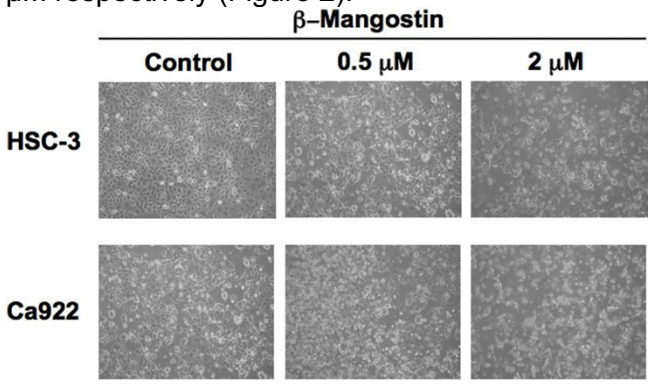

Figure 1. Morphology of the HSC-3 and Ca922 cells following exposure to β-mangostin at different concentration (0.5 μM and 2 μM) with the respective control (0.1% DMSO) after 24 h exposure. Observation was done under inverted microscope at 100X magnification.

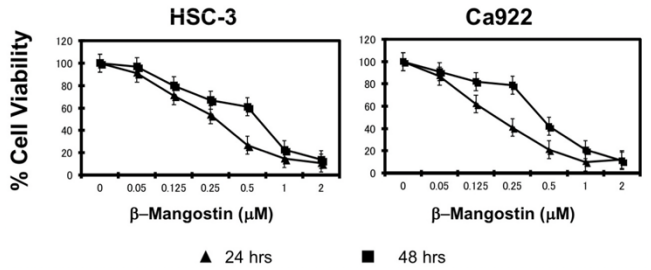

Figure 2. Dose-response curves on the effect of β-mangostin analyzed by MTT assay on HSC-3 and Ca922 cells viability at 12 or 24 h of exposure. The vertical and horizontal axes display percentage of cell viability and sample concentration (μM), respectively. Data shown are means of three independent experiments each performed in triplicates.

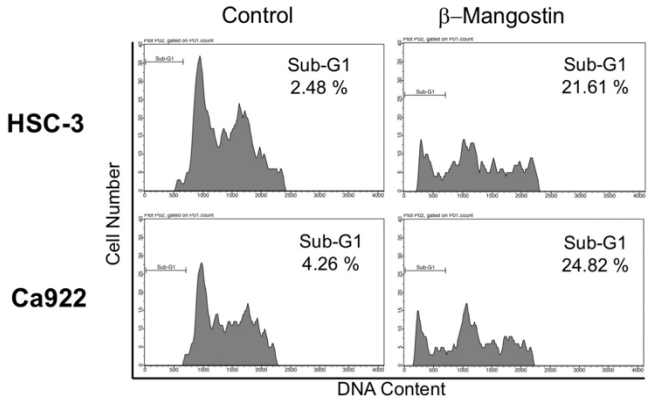

Figure 3. The cell cycle profile HSC-3 and Ca922 after 24 h treatment with either control (0.1% DMSO) or β-mangostin at 0.5 μM were analysed by flow cytometer. The horizontal and vertical axes represent DNA content and cell

number, respectively. Apoptotic cell population is indicated as a percentage of the sub-G1 fraction.

To determine the nature of cell death, cell cycle analysis using a laser scanning cytometer was performed. Apoptotic cell population is indicated as a percentage of the sub-G1 fraction. The cell cycle analysis revealed that β-mangostin treatment at 0.5 μM induced apoptosis in HSC-3 and Ca922 cells with sub-G1 population of 21.61 and 24.82 % respectively (Figure 3). Activation of caspase proteases, including caspase-3, -7, and -8 are crucial to apoptotic cell death.[23,24] Therefore, in the present study, caspase-3/7 activities assay was carried out to confirm the flow-cytometry result. Indeed, treatment with 0.5 μM β-mangostin triggered caspase-3/7 activation in both cell lines (Figure 4).

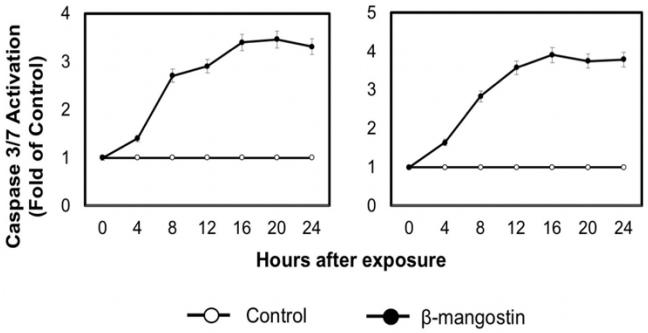

Figure 4. The caspase-3/7 activities of HSC-3 and Ca922 cell lines. The cells were treated in the same manner to those described in the flow cytometry experiment. The caspase-3/7 activity assays were conducted at specified 4 h intervals following the treatment of control or b-mangostin up to 24 hours. Relative caspase-3/7 fold-activation values depicted are means SD of sample triplicates.

The caspase 3/7 activation graph indicates an exponential increase at 4 hours interval until achieves maximum activities at 16th hour, with 3.5 and 4 fold-activation for HSC-3 and CA922 cell lines, respectively. In agreement with this, we did observe an induction in the transcript levels of caspase in HSC-3 and Ca922 after 24h of treatment by β-mangostin, as measured by quantitative RT–PCR (Figure 5), indicated that β-mangostin effects on caspase-3 and -8 activation are transcriptional. Moreover, we also observed significant upregulation of pro-apoptotic gene *PUMA* approximately 4.0-fold in both cell lines.

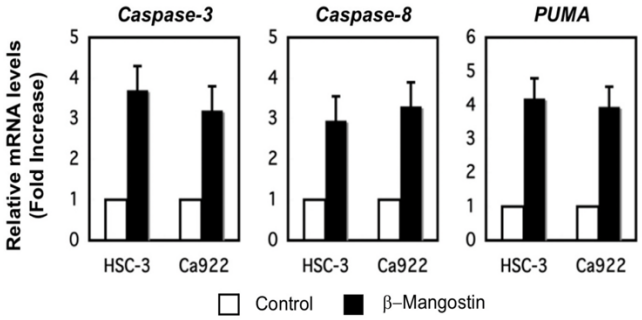

Figure 5. The relative mRNA expression of pro-apoptotic genes *Caspase-3*, *Caspase-8* and *PUMA* in HSC-3 and Ca922 cell lines using real-time PCR. Expression levels of *Caspase-3, Caspase-8,* and *PUMA* mRNAs in the cells were quantified 24 hours following treatments with either control or with 0.5 μM β-mangostin. Relative mRNA values depicted are means ± SD of two independent experiments each performed in triplicates.

Discussion

This study aimed to evaluate the effect of anticancer properties of β-mangostin on OSCC cell lines, namely HSC-3 and Ca922 cell lines. Moreover, the anticancer activities were revealed by assessing the effectiveness of β-mangostin to induce cytotoxicity, apoptosis and selected pro-apoptotic genes up regulation.

The MTT 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide, tetrazolium-based assay which introduced by Mosmann in 1983 is remain to be relevant, extensively used and acknowledged as the gold standard for determining cell viability or cytotoxicity.[25] The findings revealed β-mangostin inhibit the HSC-3 and Ca922 cells viability in a pattern of dose-dependent and time-dependent. These similar patterns were also observed, where β-mangostin had suppressed the growth of human breast cancer cells (MCF-7) in previous study.[26] The cytotoxicity activity of β-mangostin was investigated further by presenting significant the IC_{50} values.

The lower concentration was needed to yield an IC_{50} value in Ca922 than in HSC-3 cells, suggesting that Ca922 cells were relatively more sensitive toward β-mangostin. Several recent studies reported that β-mangostin exhibited potent cytotoxicity activity on the following cell

lines, with corresponding IC_{50} values: HCT116 (72.6 μM); HepG2 (97.2 μM), MCF-7 (8.9 μM), HL60 (58 μM) and DLD-1 (8.1 μM).[14,15,27]

A previous report also described β-mangostin suppressed MCF-7 cells with an IC_{50} of 26 μM and 16.5 for 24 and 48h respectively, while no significant cytotoxicity toward the normal cells (MCF10A) at highest concentration (30 μg/ml).[28]

Targeting apoptosis is the most effective non-surgical treatment, which use cell's own natural death mechanism to break down the cancerous cell's contents properly.[29] Apoptosis mechanism includes the cell shrinkage, chromatin condensation and nucleus fragmentation, followed by DNA fragmentation.[30] DNA will be fragmented into 180–200 bp sequences in length by the aid of caspase protein.[31] This study showed the percentages of apoptotic cells which contained short length fragmented DNA were markedly increased at sub-G1 phase in response to β-mangostin treatment. Nevertheless, extensive DNA fragmentation is not a comprehensive indicator in apoptotic death. Necrotic cells often display some degrees of DNA degradation that may shorten the DNA content and may be distributed across the same region of the histogram.[32] Moreover, sub-G1 cells may have relatively high DNA content and overlap with G1 cells if DNA fragmentation is not extensive during the early stage of apoptosis.[33] Therefore, caspase 3/7 assay was carried to validate the apoptotic cells detected in the flow cytometry.

Based on the literature search, β-mangostin could work on a combination of cell-cycle arrest at various G0/G1, S, G2/M phase which observed via flow cytometry and subsequently induce DNA fragmentation shown in the presence sub-G1 population various cells such as murine leukaemia cell (WEHI-3), human promyelocytic leukaemia cell line (HL60), human breast cancer cell (MCF-7) and human cervical cancer cell (HeLa).[15,26,28,34] All these studies later followed by the caspase 3/7 detection assay which revealed an increase in caspase 3/7 protein levels, confirming the apoptotic flow cytometry result.

This study has demonstrated that β-mangostin effects on caspase-3 and caspase-8 activation are transcriptional. Moreover, significant upregulation of pro-apoptotic gene *PUMA* was also observed. The expression of *PUMA* gene is low in nature and only transcriptional induction has been documented thus far. Bioinformatic studies demonstrated the possible binding site for various transcription factors which located in the PUMA gene's promoter, exon 1, and intron 1 regions.[35] p53 is one of the transcription factors that activates *PUMA*.[36] However, 50-70% of *p53* gene are mutated in OSCC.[37,38] The OSCC cell lines that being utilized in this study also had mutation at *p53* gene. Thus, others transcription factor that may transcriptionally activate *PUMA* gene are p73, p63, FOXO1, FOXO3a, CHOP, E2F1, Tribbles homolog 3, activator protein 1 (AP-1/c-Jun), C/EBPβ, cAMP response element binding protein (CREB), nuclear factor of activated T cells (NFAT), specificity protein 1 (SP1), c-MYC and interferon regulatory factor 1(IRF-1).[35] Once expressed, PUMA binds to all of the anti-apoptotic Bcl-2 members proteins and also directly activates the pro-apoptotic effectors BAX and BAK, causing MOMP, caspase cascades and cell apoptosis in various cell types.[35,39]

Caspase-8 is an initiator caspase that is tightly linked to pro-apoptotic signals and is activated via an extrinsic pathway. Caspase-8 is activated by the presence of different receptor/ligand pairs. Once activated, caspase-8 cleaves and activates downstream effector caspases such as caspase-3, -6, and -7, demonstrating the importance of caspase-8 in the apoptosis pathway.[40] Previous research reported that β-mangostin demonstrated the upregulation of caspase-3 and caspase-8 during apoptosis detection analysis in human breast cancer cell (MCF7).[28] A previous report also described β-mangostin influenced the upregulation of *Bax, caspase-3*, and *caspase-9* genes in apoptosis inducing experiment using human promyelocytic leukaemia cell line (HL60).[15] This study suggests that induction of apoptosis in HSC-3 and Ca922 by β-mangostin linked with the activation of *caspase-3, caspase-8* and pro-apoptotic *PUMA* gene.

Conclusions

From this study, we conclude that β-mangostin could inhibit cell growth, induce apoptosis and upregulate pro-apoptotic gene expression such as *Caspase-3*, *Caspase-8* and *PUMA* in both HSC-3 and Ca922 cell lines. β-mangostin could be one of the significant

anticancer candidates inducing apoptosis-targeting pathway. These findings will give insight into the promising use of β-mangostin as alternative plant natural remedies in OSCC management.

Declaration of Interest

The authors declare that there is no conflict of interest.

Acknowledgements

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