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Obacunone and obacunone glucoside inhibit human colon cancer (SW480) cells by the induction of apoptosis

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ABSTRACT

Objectives: The study was aimed to purify obacunone and obacunone glucoside (OG) from seeds of Marsh White grapefruit and understand the mode of cytotoxicity of limonoids on colon cancer (SW480) cells. Methodology: Both limonoids were purified using chromatographic techniques. The structures and purity of limonoids were confirmed by NMR and HPLC analysis, respectively. Results: Obacunone and OG inhibited SW480 cell proliferation with IC_{50} values of 97 and 109.7 μM respectively, at 24 h. Sequence of events such as decreased ratio of *bcl2*/*bax* gene transcription, activation of caspase-3, fragmentation of DNA in cells treated with obacunone and OG demonstrated induction of apoptosis by limonoids. Additionally, higher induction of cytochrome-c in cytosol suggests the activation of intrinsic apoptosis by limonoids. Involvement of apoptosis was also confirmed through expression of bax, bcl2, pro-caspase-3 and caspase-9. Both the limonoids activated p21 and arrested cells at G1 and G2/M phase. Additive activity of proliferation inhibition and activation of caspase-3 by limonoids was observed when combined with camptothecin, demonstrating the induction of apoptosis. In conclusion, both limonoids induced apoptosis by activation of intrinsic apoptosis pathway and activation of p21 leading to arresting cells at G2/M phase of the cell cycle.

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1. Introduction

Colorectal cancer accounts for over 90% of the malignant tumors of the large bowel and remaining 10% consist of lymphoma and squamous cell carcinoma (Greenstein et al., 1989). Colorectal cancer is the second most common cause of death from malignant disease in US and the western world. Several epidemiological studies have demonstrated the association of colon cancer with dietary habits such as low fiber intake, high fat diet, and low calcium/micronutrient intake (Johnson and Mukhtar, 2007). An inverse relation between consumption of fruits and vegetables with incidence of various cancers has lead researchers to investigate the benefits of dietary components in cancer prevention mechanism (Dragsted et al., 1993). Results of *in vivo* and clinical studies suggest that bioactive compounds of fruits and vegetables (citrus is included as one of the component in these studies) may help in the prevention of colon cancer (Michels et al., 2000; Nangia-Makker et al., 2002; Steinmetz and Potter, 1996).

Citrus fruits contain myriad of biologically beneficial, non-nutritive compounds such as, flavonoids, terpenoids, phytosterols, folate, pectin and coumarins. Our group has reported the isolation, identification of citrus bioactive compounds and demonstrated proliferation inhibition of different cancer cells by various limonoids (Jayaprakasha et al., 2008; Jayaprakasha and Patil, 2007a; Poulose et al., 2007). Among the limonoids, obacunone and OG (Fig. 1A) are found in most of the citrus fruits in minor quantities. Commercial citrus juice contains 3.35–4.25 μg/mL of OG and the trace amounts of obacunone (Herman et al., 1990). However, seeds of mandarin contain upto 1.3 ± 0.6 mg/g of obacunone and 1.8 ± 1.0 mg/g of OG (Ozaki et al., 1991).

Proliferation inhibition and suppression of carcinogen induced tumor by citrus limoniod were reported by several studies in the past (Lam et al., 1989; Tanaka et al., 2000b). Obacunone potentiated the cytotoxicity of microtubule inhibitor drugs in L1012 (mouse lymphocytic leukemia) and drug resistant KB-3-1 cells (Jung et al., 2000). Another feeding study performed using obacunone was demonstrated elevation of GST (glutathione S-transferase) level of liver, forestomach, lung and colon (Lam et al., 1989) in experimental mice. The GST enzyme is known to detoxify carcinogens and other toxic metabolites responsible for initiation of cancer. Induction of phase-II enzymes were also observed in carcinogen induced colon cancer model (Tanaka et al., 2000b). Studies from our laboratory has demonstrated differential inhibition of proliferation of blood (HL-60), ovary (SKOV-3), cervix, stomach (NCI-SNU-1), liver (Hep G2) and breast (MCF-7) carcinoma cells

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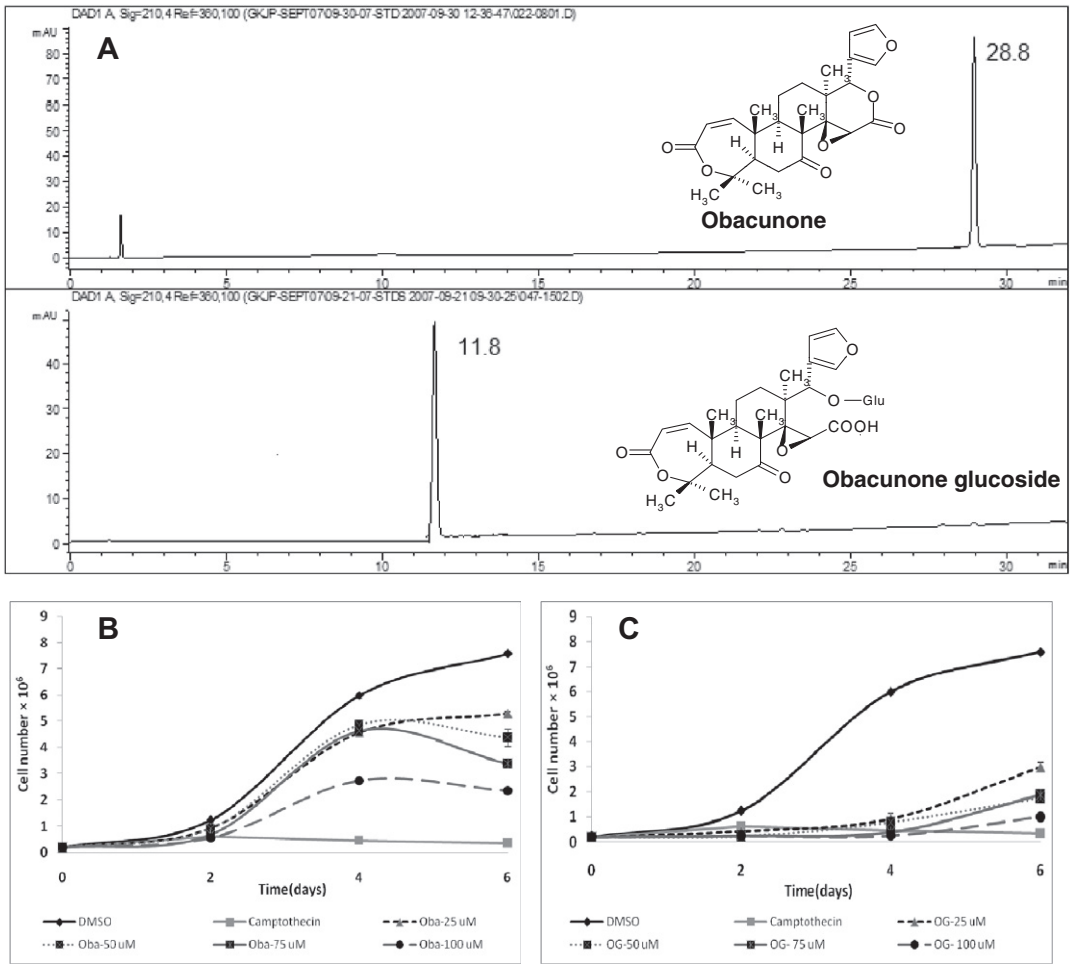

Fig. 1. HPLC chromatograms of obacunone and OG and their effect on cell viability, (A). Separation of compound 1 and 2 were carried out on reverse phase C_{18} column (250 × 3.1 mm i.d. with particle size of 5.0 μ). Gradient elution was performed by 3.0 mM H_3PO_4 and acetonitrile. The chemical structures of the obacunone and OG are inserted in the chromatograms. (B). Effect of obacunone and (C). OG on viability of SW480 cells. (Values are Mean ± SD, $n = 9$). Cell counting assay was performed using Beckman coulter Z_1 as explained in methods.

by different limonoids (Tian et al., 2001). Another study from our laboratory has demonstrated that OG inhibits neuroblastoma cells at 50 μM and lower concentrations. This inhibition ability of OG was attributed to reduction in DNA synthesis and induction of caspase 3/7 activity (Poulose et al., 2005,2006).

Although numerous reports published on the ability of citrus limonoids to inhibit proliferation and tumor markers in certain cancers, very little information is available on possible mechanism of inhibition using pure limonoids. The current study reports the isolation, identification of two limonoids and possible mechanism of cytotoxicity using colon cancer (SW480) cells.

2. Materials and methods

2.1. Chemicals and reagents

Dowex-50, silica gel and solvents were purchased from Sigma–Aldrich (St. Louis, MO, USA). The SP-70 adsorbent resin was purchased from Supelco (Bellefonte, PA, USA). Marsh White grapefruits (*Citrus paradisi* Macf.) were collected from the Texas A&M University-Kingsville (Citrus Center, Weslaco, TX, USA). Seeds were separated manually and finely powdered. The ^{13}C NMR spectra were recorded at 100 MHz respectively on a JEOL NMR (JEOL USA Inc., MA, USA) instrument, tetramethylsilane (TMS) was used as an internal standard. Fluorescent probes were purchased from Invitrogen molecular probes (Carlsbad, CA, USA). Media and chemicals for cell culture were obtained from Hyclone cell culture and bio-processing (Logan, UT, USA). Both primary and secondary antibodies used in the study were procured from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary and secondary antibody for Cleaved caspase-3 antibody was purchased from cell signaling technologies (Danvers, MA, USA). Unless otherwise mentioned, control refers to the SW480 cells treated with DMSO equal to that of sample.

2.2. Cell culture and maintenance

Both SW480 (colon cancer) and NIH 3T3 (mouse embryo fibroblast) cells were obtained from ATCC (American Type Culture Collection, Manassas, USA) and cultured in DMEM containing streptomycin and penicillin (100 units of each) with 10% fetal bovine serum. Cells attaining 70% confluence with normal morphological features were used for the experiments. Cells were maintained in a sterile biological incubator, maintained at 37 °C with 95% air and 5% carbon dioxide with 85 ± 5% relative humidity. All the experiments were performed as three biological replicates with minimum of three independent experiments for each compound, concentration, and time point.

2.3. Extraction and purification

Powdered Marsh White grapefruit seeds (5.6 kg) were defatted with hexane and extracted with 15 L of ethyl acetate and methanol in Soxhlet extractor for 16 h each at 60 °C, successively. The extracts were filtered and concentrated under vacuum separately to obtain viscous liquid. Ethyl acetate extract (45 g) was impregnated with silica gel (30 g) and loaded onto 250 g of silica gel. The column was eluted with different ratio of hexane and hexane: chloroform. Elution with hexane: chloroform (60:40) provided 38 mg of compound 1. The methanol extract was purified repeatedly as per our published method (Jayaprakasha et al., 2007b) to obtain 180 mg of compound 2.

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