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Aromadendrene oxide 2, induces apoptosis in skin epidermoid cancer cells through ROS mediated mitochondrial pathway

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ABSTRACT

Aims: Aromadendrene oxide 2 (AO-(2)) is an oxygenated sesquiterpene naturally found as a chemical component of essential oils. In the present study anticancer activity of AO-(2) has been investigated on A431 human epidermoid cancer and precancerous HaCaT cells.

Material and methods: Cell viability was used to detect cytotoxic activity. Mechanism of cell death induced by AO-(2) treatments was studied using Annexin V-FITC/PI binding, cell cycle analysis, measurement of MMP and ROS generation by flow cytometry. Expression of apoptosis related proteins was investigated by western blot.

Key findings: AO-(2) inhibited the growth and colony formation ability of A431 and HaCaT cells in concentration dependent manner. It induced cell cycle arrest at G0/G1 phase and apoptosis through intracellular ROS accumulation. Inhibition of intracellular ROS by ascorbic acid and *N*-acetyl cysteine treatment completely blocked apoptotic effect. *N*-acetyl cysteine treatment significantly reversed G0/G1 arrest induced by AO-(2). AO-(2) treatment caused loss of mitochondrial membrane potential ($\Delta\Psi m$), increase in Bax/Bcl-2 ratios, cytochrome *c* release, activation of caspases (cleaved caspase-3 and caspase-9) and PARP cleavage. AO-(2) also significantly inhibited the growth of multicellular tumor spheroids of A431 and HaCaT cells.

Significance: The results of the present study reveals that AO-(2) a chemical component of essential oils induces apoptosis in A431 and HaCaT cells.

1. Introduction

The incidence of non-melanoma skin cancer (NMSC), such as basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) is a rising problem in most countries [1,2]. Exposure to UV radiation has been found to be the major etiological factor leading to the precancerous stage of actinic keratosis and the development of skin malignancies [3,4]. BCC, which accounts for 80–85% of all NMSCs, hardly metastasizes to other organs [5,6] while SCC, which accounts for 15–20% of all NMSCs, is more likely to invade other tissues and can cause death [5,7]. The growing incidence of cutaneous malignancies indicates the need for multiple treatment options. Although surgical modalities remain the basis of treatment, effective strategies are still required to reduce morbidity and mortality. Therefore, development of effective chemotherapeutic agents and complementary approaches are needed to treat skin carcinoma. Numerous evidences reported that apoptosis induction is considered to be one of the promising therapeutic strategies against cancer [8]. One such approach is to utilize the potential of natural products, especially those derived from plants, to be effective in cancer chemoprevention and cancer therapy [9]. Natural products and their derivatives are important sources of novel therapeutic molecules [10]. Plant essential oils are complex mixtures of components such as monoterpenes, sesquiterpenes, alcohols, ethers, aldehydes, esters and ketones [11]. Of these, monoterpenes, sesquiterpenes and oxygenated derivatives are the largest group of chemical entities in EOs [12]. In the recent years EOs and their major components like Carvacrol, d limonene, Geraniol, Myrcene, perillyl alcohol, α-humulene, Thymol, Citral and others have also been reported to possess cytotoxic effect against cancer cell lines and in vivo studies [13]. Aromadendrene oxide 2 AO-(2) is an oxygenated sesquiterpene naturally found as a component in EOs of plants. We have identified Aromadendrene oxide 2 AO-(2) as a component (14.1%) of essential oil from *P. missionis* (Wight) Swingle (Rutaceae) by GC–MS analysis. The anticancer activity of *P. missionis* essential oil in A431 skin epidermoid cancer cells and precancerous HaCaT cells has been reported in our previous study [14]. To our knowledge there are no reports on anticancer activity of Aromadendrene oxide 2. The present work examines the underlying mechanism for the observed cytotoxicity induced by Aromadendrene oxide 2 in

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A431 (human epidermoid skin carcinoma) and precancerous HaCaT cells.

2. Material and methods

2.1. Chemicals and antibodies

Aromadendrene oxide 2 (> 98% purity) was obtained from Sigma Aldrich. 3-(4,5-dimethyl thiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) (SRL), propidium iodide (PI), 2,7-dichlorofluorescein diacetate (DCFH-DA), Ascorbic acid (AA), *N*-acetyl cysteine (NAC) and β-actin antibody were purchased from Sigma-Aldrich, USA. Annexin FITC/PI kit and 5,5′,6,6′-tetrachloro-1,1′,3,3′ tetra ethyl benzimidazolyl carbocyanine iodide dye (JC-1), were procured from Abcam technologies, and RNAse A from MP Biomedical. Anti-caspase-3 (cleaved) and anti-caspase-9 (cleaved) were obtained from Cell Signalling Technology, anti-caspase-8 from Santa Cruz Biotechnology, anti-Bax from Millipore and anti-Cyclin D from BD Biosciences. Anti-cytochrome C, anti-Bcl-2 and anti-PARP were procured from Abcam Technologies.

2.2. Cell culture

Human epidermoid skin carcinoma A431 and precancerous HaCaT cells were obtained from National Centre for Cell Science, Pune, India. A431 and HaCaT cells were grown and maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine (200 mM), 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in 5% CO_2 incubator. Human mesenchymal stem cells (HMSCs) (isolated from umbilical cord) were cultured in alpha MEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine (200 mM), 100 U/ml penicillin and 100 µg/ml streptomycin. All the cells were maintained in a humidified 5% CO_2 incubator at 37 °C.

2.3. Cytotoxic activity

Cytotoxicity of AO-(2) on A431 and precancerous HaCaT cells were assessed by MTT assay [15]. Stock solutions of AO-(2) (100 mM) was prepared in absolute ethanol. Stock solution was further diluted to make desired final concentrations with growth medium just before use. Briefly, cells at exponential growth phase were taken up for the study. 5000 cells per well were seeded in 96-well plate. The cells were allowed to attach (overnight), and on the next day fresh culture medium containing increasing concentrations of AO-(2) ranging from 10 to 200 µM were added. 5-Fluorouracil (5-FU) was used as a positive control drug in present study. Plates were incubated for 24, 48 and 72 h. After the incubation period 10 µl of MTT (5 mg/ml) was added to the cells and further incubated at 37 °C in 5% Co2 incubator for 3 h. The MTT-formazon product formed was dissolved in 10 µl of stop solution (20% SDS in 50% dimethylformamide). Absorbance was measured at 570 nm (with reference at 620 nm) in an ELISA plate reader. The percent of cell viability was determined by using the following formula, % Cell viability = (Absorbance of treated cells / Absorbance of untreated cells) * 100. All experiments were repeated at least three times independently.

2.4. Colony formation assay

A431 and HaCaT cells (1000 cells/well) were seeded in 6-well plate, allowed attach and exposed to increasing concentrations of AO-(2) for period of 72 h. After incubation period the medium was replaced with fresh medium and surviving cells were allowed to grow and form colonies for 14 days (colony containing 50 cells). Colonies were fixed in ice cold methanol and stained with 0.5% crystal violet stain [16]. Excess stain was removed by washing plates in water. Plates were air dried and Photographed. Stained colonies in the control and treated cells were counted by using Image J software. Percentage colonies formed were calculated using the formula: (Number of colonies formed by treated cells) / (Number of colonies formed by untreated cells) * 100. Experiments were repeated three independent times.

2.5. Multicellular Cancer Tumor spheroids

Multicellular Cancer Tumor spheroids (MCTS) show high resemblance to solid tumors. Cytotoxic activity of AO-(2) was tested on MCTS. Multicellular Cancer Tumor spheroids were generated as described previously [17,18] with slight modifications. Spheroids were produced by seeding cells in 96 well plates pre coated with 2% w/v agar. Briefly A431 cells at density of 25,000/well, HaCaT cells at density of 50,000/well were seeded in 200 µl of RPMI media containing 10% FBS in 96 well plates. Cells were allowed to aggregate and form spheres for period of 48 h. 3D spheroids of uniform size (350–400 µm) were exposed to fresh media containing increasing concentrations of AO-(2) for 96 h at 37 °C in a 5% CO_2 incubator. Spheroid viability was determined by MTT assay at 96 h. Spheroid size reduction upon treatment with different concentrations of AO-(2) was measured using Nikon Ti pad software.

2.6. Acridine orange-ethidium bromide (AO/EtBr) staining

Cells undergoing morphological and nuclear changes upon treatment with AO-(2) were detected by AO/EtBr staining by fluorescence microscopy [19]. 5000 cells were seeded in 96-well plate, allowed to attach for overnight period and treated with AO-(2) (10–200 µM) for 72 h. After incubation period cells were stained with 2 µl of the AO/EtBr (Sigma-Aldrich, USA) dye mix (100 µg/ml) (1:1) and incubated in dark for 15 min. Cells were visualized immediately and photographed under fluorescence microscope (Nikon Eclipse Ti).

2.7. Cell cycle analysis by flow cytometry analysis

The progression of the cell cycle was determined by flow cytometry. Briefly 1×10^5 exponentially growing cells were seeded in six-well culture plates and were allowed to adhere overnight. Next day the medium was replaced and cells were exposed to increasing concentrations of AO-(2) for 24, 48 and 72 h. After the incubation period untreated and treated cells were collected, washed with PBS and fixed in 70% ethanol for overnight period at 4 °C. Fixed cells were washed with PBS to remove residual ethanol. Cells were re-suspended in 300 µl of PBS containing propidium iodide (PI) (50 µg/ml), 0.5% Triton X-100 and incubated with RNAse A (50 µg/ml) at 37 °C for 30 min in water bath [20]. Cells were analyzed for cell cycle distribution at different phases (G0/G1, S, G2/M and sub-G1) in FACS Calibur flow cytometer (BD Biosciences, USA).

2.8. Detection of apoptosis by Annexin V FITC assay

Apoptosis induction was analyzed by flow cytometry using Annexin-FITC/PI kit (Abcam, U.K). 1×10^5 cells/ml were treated with increasing concentrations of AO-(2) for period of 72 h. After incubation cells were washed with PBS and suspended in 300 µl of binding buffer and stained with Annexin V-FITC (1.5 µl) antibody, propidium iodide (1.5 µl) and incubated in dark for 5 min. Stained cells were immediately analyzed by flow cytometry (FACS Calibur),using quadrant statistics for sorting out live, early apoptotic, late apoptotic and necrotic cell populations.

2.9. Measurement of mitochondrial membrane potential

Changes in the mitochondrial membrane potential was assessed using JC-1 dye. Briefly, cells were incubated with the increasing concentrations of AO-(2) for 72 h in 24-well plate. Then the cells were

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