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[6]-Shogaol attenuates inflammation, cell proliferation via modulate NF-κB and AP-1 oncogenic signaling in 7,12-dimethylbenz[*a*]anthracene induced oral carcinogenesis

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ABSTRACT

Nuclear factor-kappaB (NF-κB) and activator protein 1 (AP-1) is a major transcription factor which regulates many biological and pathological processes such as inflammation and cell proliferation, which are major implicates in cancer progression.

[6]-Shogaol ([6]-SHO) is a major constituent of ginger, exhibits various biological properties such as antioxidants, anti-inflammation and anti-tumor. Recently, we proven that [6]-SHO prevents oral squamous cell carcinoma by activating proapoptotic factors in *in vitro* and *in vivo* experimental model. However, the preventive efficacy of [6]-SHO in 7,12-dimethylbenz[*a*]anthracene (DMBA) induced hamster buccal pouch carcinogenesis (HBP) has not been fully elucidated, so far. Hence, we aimed to investigate the effect of [6]-SHO on inflammation and cell proliferation by inhibiting the translocation of NF-κB and AP-1 in DMBA induced HBP carcinogenesis. In this study, we observed upregulation of inflammatory markers (COX-2, iNOS, TNF-α, interleukin-1 and -6), cell proliferative markers (Cyclin D1, PCNA and Ki-67) and aberrant activation of NF-κB, AP-1, IKKβ, c-jun, c-fos and decreased IκB-α in DMBA induced hamsters. Conversely, oral administration of [6]-SHO strongly inhibited constitutive phosphorylation and degradation of IκB and inhibit phosphorylation of c-jun, c-fos, resulting in inhibition of nuclear translocation of NF-κBp65 and AP-1. Thus, inhibition of NF-κB and AP-1 activation by [6]-SHO attenuates inflammation and cell proliferative response in DMBA induced hamsters. Our finding suggested that [6]-SHO is a novel functional agent capable of preventing DMBA induced inflammation and cell proliferation associated tumorigenesis by modulating multiple signalling molecules.

1. Introduction

The global burden of cancer continues to increasing largely, especially oral cancer. It is estimated that incidence 3, 00, 373(2.1%), mortality 1, 45, 328(1.8%) and 5-year survival 7, 02, 149(2.2%) in oral cavity cancer. In India, estimated that incidence 77,003 (7.6%), mortality 52,067 (7.6%), 5-year prevalence 118,902 (6.6%) according to data of GLOBOCAN 2012 [44]. The multistep process of oncogenesis characterized by the defective genome of inflammation, cell proliferation and apoptosis on the cellular and genetic level that ultimately reprogram a cell to undergo uncontrolled cell proliferation has been suspected in tumor progression. These pathophysiological processes linking with several signal transduction molecules such as NF-κB and AP-1 is the major causative role in an inflammation, cell proliferation and cancer development [1]. NF-κB, a redox-sensitive transcription factor tightly associated with inhibitor of kappa B (IκB) sequestered in the cytoplasm under the neutral state. The rapid phosphorylation and proteolysis of IκB allows to the activation of NF-κB. Thus, free NF-κB subsequently translocated into the nucleus and bind to specific response elements in DNA sequence sites for gene transcription [2,3]. Several experimental models of cancer in which NF-κB and AP-1 activation have been inhibited by dietary agents are crucial for the cancer therapeutic drug development [4].

Cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) are key molecules for the inflammatory process. These inflammatory mediators were upregulated in human oral squamous cell carcinoma (SCC) [5,6]. Phytochemicals could inhibit COX-2 and iNOS expression in the DMBA induced oral carcinogenesis provides a rationale for the development of therapeutic drugs that may be useful for various inflammatory diseases [7]. Proliferating cell nuclear antigen (PCNA), Cyclin D1 and Ki-67 are major cell proliferative markers which can be used for understanding the cell cycle progression and tumor development, as well as being used as a biomarker for cancer diagnosis and prognosis [8]. PCNA, an non-histamine nuclear protein of DNA

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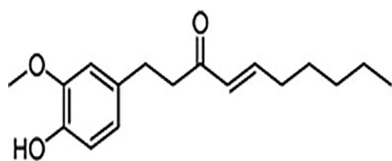

Fig. 1. Chemical structure of [6]-shogaol.

polymerase δ plays an important role in cell proliferation and cell cycle regulation [9]. Cyclin D1 is another nuclear protein involved in the transition from G1/S phase of the cell cycle. Ki-67 is an admirable diagnostic marker in cancer and it is detected throughout all phase of the cell cycle with the exception of early G1 phase [10]. This protein is frequently amplified and over-expressed in patients with oral squamous cell carcinoma and experimental model [11].

Ginger *(Zingiber officinale)* has been widely used as a spice and medicinal herb in traditional herbal medicine. [6]-SHO (Fig. 1a) is a natural dietary phenolic ingredient of ginger with several pharmacological properties such as anti-inflammatory, anti-cell proliferative and anti-tumour [12–14]. Additionally, Tan et al. [14] documented that

[6]-SHO induces apoptosis and regulate cell cycle through activation of peroxisomal proliferator activated receptor γ (PPAR γ) in breast and colon cancer cells. Notably, Pan et al. [13] reported that [6]-SHO could inhibit pro-inflammatory (iNOS, COX-2) gene expression and NF-κB transcription activation. We previously reported that [6]-SHO induced apoptosis in DMBA induced hamsters and *in vitro* model [15,16]. However, the molecular mechanisms underlying the chemopreventive efficacy of [6]-SHO remain largely unknown. In the present study, we report that [6]-SHO inhibits DMBA induced inflammation and cell proliferation in hamster buccal pouch by suppressing NF-κB and AP-1 signaling. These investigations are apparently to be helpful for identifying novel chemotherapeutic drugs for the treatment of oral cancer.

2. Materials and methods

2.1. Reagents

7,12 dimethylbenz (a) anthracene (DMBA), Sodium dodecyl sulphate (SDS), Acrylamide, *N,N,N′,N′*-tetramethylene diamine (TEMED), 2-mercaptoethanol, bromophenol blue, bovine serum albumin (BSA), hydrogen peroxide, ethidium bromide (EtBr), low-melting point agarose (LMPA), primary anti-monoclonal antibodies such as tumour necrosis factor-α (TNF-α), interleukin -1 (IL-1), interleukin -6 (IL-6), iNOS, COX-2, Cyclin-D1, PCNA, Ki-67, β-actin and Oligo primers were purchased from Sigma-Aldrich, USA. [6]-Shogaol > 95% purity by High-performance liquid chromatography (HPLC) (CAS No. 555-66-8) was purchased from Natural Remedies, Bangalore.

2.2. Animals

A total of 40 male golden Syrian hamsters aged 8–10 weeks, were obtained from the National Institute of Nutrition (NIN), Hyderabad, India and maintained in polypropylene cages under standard conditions of temperature and humidity with an alternating 12-h light/dark cycle and provided standard pellet diet (Mysore Snack Feed Ltd, Mysore, India). The experiments were carried out according to the guidelines approved by Institutional Animal Ethics Committee of Animal care (IAEC), Annamalai University (No.160/1999/CPCSEA/10801).

2.3. Experimental design

A total of 40 hamsters were randomized as control and experimental groups and divided into 4 groups of ten hamsters each. In group 1, control hamsters received liquid paraffin alone. In group 2 and 3, the right buccal pouches of hamsters were painted with DMBA (0.5%) in liquid paraffin using a No. 4 sable brush, thrice a week for 16 weeks [17]. In group 2 DMBA induced hamsters received no other treatment. Groups 3 hamsters painted with DMBA, additionally, received [6]-SHO at a dose of 20 mg/kg body.weight, thrice a week on alternate days to DMBA application. In group 4, hamsters were received [6]-SHO (20 mg/kg b.wt) alone. The experiment was terminated at the end of 16th weeks and all animals were sacrificed by cervical dislocation after an overnight fasting.

2.4. Immunohistochemical (IHC) staining

The immunohistochemical expression of PCNA, Cyclin D1 and Ki-67 were performed on 5 μm paraffin embedded tissue section on poly-L-lysine coated glass slides. In brief, frozen buccal tissue/tumor sections (5 μm) were cut using cryostat (Slee, Mainz, Germany) and the tissue sections were fixing the slides in an oven at 60 °C for 10 min and then rinsed with xylene twice for 5 min. Each slide was hydrated in graded ethanol series (100%, 90%, 70%, 50% and 30%) for 10 min and then finally rinsed in double distilled water for 3–5 min. The sections were incubated with 1% H_2O_2 to quench endogenous peroxidase activity for 10 min at 22 °C. After incubation, slides were washed three times in Tris buffer containing 150 mM NaCl (pH 7.4) for 10 min and blocked in blocking buffer (1 X TBS, 5% NFDM, 0.05% Tween) for 1 h at 22 °C. After washing with 1 X TBS containing 0.05% Tween 20, the sections were incubated with respective antibodies PCNA, Cyclin D1 and Ki-67 (Sigma Aldrich, USA) at a dilution of 1:3000 respectively, overnight at 4 °C. After incubation, the sections were again rinsed with 1 X TBS buffer and were incubated with secondary antibody, goat anti-rabbit IgG-HRP conjugate (Sigma Aldrich, USA), at a dilution of 1:3000, for 1 h at 4 °C. The color was developed using chromagen 3-methyl 9-ethyl carbazole in Tris (0.05 M, pH 7.3) buffer containing H_2O_2.The immunostained slides were analyzed by under bright field Olympus microscope [18].

2.5. Immunoblot analysis

The cytoplasmic and nuclear extracts were prepared by the method of Legrand-Poels et al. [41] with modification. Tissue samples were homogenized with 1 ml of homogenizing buffer containing 10 mM HEPES (pH 7.9), 0.5 mM PMSF, 0.1 mM EGTA, 1 mM DTT, 0.1 mM EDTA, 10 mM KCl and 10 μL protease inhibitor cocktail. The tissue lysate was then centrifuged at 8000 × *g* for 2 min at 4 °C, the cytoplasmic supernatant was removed, and aliquoted was stored at −80 °C. The nuclear pellet was reconstituted in 1 ml of buffer containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM DTT, 1 mM EGTA and 10 μL protease inhibitor cocktail, followed by hearty vortexing for 25 min at 4 °C. The nuclear lysate was then centrifuged at 14,000 × *g* for 5 min, and nuclear extracts were aliquoted and stored at −80 °C for further analysis. The Proteins were fractionated by using 10% SDS-PAGE gel electrophoresis and the resolved proteins were blotted onto PVDF membrane and probed with a primary monoclonal antibody such as TNF-α, NF-κB p65, IκB, c-Jun, c-Fos, AP-1, COX-2, iNOS, PCNA, Cyclin-D1 and Ki-67 as per the method described previously [16].

2.6. RNA isolation

Total RNA was extracted from control and experimental hamster's buccal tissues parts by using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. The amount of total RNA was quantified by using Nanodrop 2000 (Thermo Scientific, USA). Further, the integrity of the RNA was verified by electrophoresis on denaturing agarose gel.

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