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Daphnetin ameliorates 7,12-dimethylbenz[a]anthracene-induced mammary carcinogenesis through Nrf-2-Keap1 and NF-κB pathways

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ABSTRACT

Cancer is a faction of disorders that conjugated primarily with oxidative imbalance. In mammary carcinoma, oxidative stress secondarily changes various gene expressions and signalling pathways that bring genomic instability and mutagenic alterations that fascinating carcinogenesis. Several coumarin compounds are active against various malignancies. Among them, daphnetin (DAP) exhibits valuable safety and bioactivity profile that contributes towards its efficacy against cancer. In this study, the antioxidative and chemotherapeutic potential of DAP against 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary carcinogenesis was evaluated in female Sprague-Dawley rats. Besides this, we have determined the effect of DAP on Keap1-Nrf-2, associated HO-1 and NF-κB expressions behind the antioxidative and anti-proliferating activity. In our findings, a protective effect of DAP was established against lipid peroxidation, enzymic (Total SOD, MnSOD, CuZnSOD, CAT, GPx) and non-enzymic (GSH) antioxidative markers in serum, liver, kidney and breast tissue of both control and experimental groups. An up-regulation of protective Nrf-2 & HO-1 with a synchronized suppression in Keap1 & NF-κB mRNA and protein expressions were observed. DAP revealed the inhibition of p-AKT which accountable for decrease in NF-κB expressions but shown to be ineffective on p-ERK1/2. This study revealed that DAP inhibits mammary carcinogenesis through multiple mechanisms. Dual efficacy of DAP on Nrf-2-Keap1 pathway and NF-κB expressions propose it as a potential chemotherapeutic agent in mammary cancer management.

1. Introduction

Cancer became a major public health concern with enhanced carcinogenic exposures that cumulatively increase cancer risk. Among these, mammary carcinoma is one of the most serious problems in developed as well as developing countries. Mammary carcinogenesis is characterized by cellular and physiological changes followed by formation of a lump or tumour. It is well established that oxidative imbalance is an imperative contributing factor in carcinogenesis of mammary carcinoma [1,2]. Apart from various beneficial roles in physiological functioning, free radicals or reactive oxygen species (ROS) accumulation may bring structural and physiological changes to normal cell biology which further may precipitate many deteriorative damages leading to certain pathologies like cancer [3]. This oxidative misbalance regulates the expression of various important gene and proteins modulating different signalling pathways and process such as cell growth, angiogenesis, apoptosis, proliferation, invasion and DNA repair, etc. [3,4]. This multi-dysfunction brings genomic instability and mutagenic changes to various cell organelles like mitochondrion, nucleus, endoplasmic reticulum, etc. that lead to the development of cancer. Many studies reported a comparatively higher concentration of ROS production in cancer cells [3,5]. These free radicals may act as a promoter to cancer initiation, proliferation, and its progression.

The endogenous disturbance in oxidative balance leads to activation of antioxidative repair mechanisms and defence systems mediated by certain specific signalling pathways like NF-κB (Nuclear Factor-kappa B), Keap-1-Nrf-2-ARE (Kelch-like ECH-associated protein 1-nuclear factor erythroid2 (NF-E2)-related factor 2-antioxidant responsive element) pathways. These cascade of events involved many enzymes and factors that may have paradoxical effects or counteract the changes occurred due to oxidative reactions [6]. Evidence reported up-regulation of Keap-1 and simultaneous down-regulation of Nrf-2 expression that enhancing the cellular susceptibility towards ROS, which further

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potentiate the immune or inflammatory progression in the process of carcinogenesis [7]. These changes evident to be resulted in over-expressions and up-regulations of inflammatory genes like NFAT, NF-κB-subunits which then freely induce ample of genes promoting malignancy and metastasis [8]. Further, carcinogenesis mediated modulation of Nrf-2 gene directly and indirectly (other immune responses) activated NF-κB mediated tumorigenesis progression in breast carcinoma [9]. Thus, inhibition of Keap-1, NF-κB gene expressions and induction of Nrf-2 and related antioxidants may contribute to enhance the chemotherapeutic and cytoprotective potentials of the anticancer moiety. These changes can specifically be determined by the change in oxidative marker enzymes in mammary carcinoma tissues [1,2] as the biochemical endpoint of chemotherapeutics.

Phytochemicals are the most accepted alternative and adjuvant medicines in several pathologies like cancer. Among all, coumarins embrace one of the most assessed and studied natural phenolic compounds having a variety of biological activities and nutritive values as well [10]. Various coumarin compounds are in clinical uses in the management of thrombotic disorders, chronic infections, thermal injuries and immunological disorders. Moreover, coumarins also have been appraised from few decades for the management of various malignancies including, hepatotoxicity, renal cell carcinoma, mammary carcinoma and malignant melanoma [11,12]. Daphnetin (DAP), a 7,8-dihydroxylated derivative of coumarin is a secondary metabolite of various plants used as a conventional medication. Notably, DAP possesses anticoagulant, anti-arthritic, antipyretic, and immunosuppressive potentials [13,14]. DAP expresses antiproliferative and chemotherapeutic activity against various carcinoma *in-vivo* and *in-vitro* models [15,16]. It also modulates several intermediate signalling pathways and their mediators like protein kinase C, NFAT, NF-κB, Keap1, which leads to significant chemotherapeutic and anti-proliferative activity among malignant cell lines [13,15,17]. Although, DAP ameliorate oxidative stress-related hepatotoxicity via activating Nrf2-mediated HO-1 expression, [10] the multi-mechanistic approaches of chemotherapeutic potentials of DAP is ambiguous to date. DAP gives very favourable antioxidative, and bioactive profile and its antiproliferative activity against MCF-7 cells showed that it may be a competent anti-tumoral agent acting on estrogen-dependent tumours [15]. In this study, we designed to investigate the antioxidant and chemotherapeutic potential of DAP against DMBA-induced mammary carcinogenesis in female Sprague-Dawley (SD) rats and tried to evaluate its' underlying molecular mechanisms.

2. Materials and methods

2.1. Experimental animals

Female SD rats (50–55 days old) were used in this study. Animals were purchased from National Institute of Nutrition (NIN), Hyderabad, India, and maintained in Central Animal Facility, Birla Institute of Technology, Mesra, Ranchi, India (Reg. No. 621/02/ac/CPCSEA) and the study protocol was approved by IAEC (Institutional Animal Ethics Committee; Protocol Approval No. BIT/PH/IAEC/24/2013). Habitat and victuals of all animals were maintained according to standard guidelines. The animals were acclimatized to the laboratory conditions for a week before the commencement of the experiment. All experiments involving animals complies with the ethical standards of animals handling.

2.2. Carcinogenesis induction and treatment protocol

Mammary tumours were induced by DMBA using the "air-pouch technique" as described [18] with small modifications as required. Briefly, about 2–3 ml of sterile air was carefully injected subcutaneously just beneath the mammary fat pad region of the 2nd and 3rd mammary glands so as to produce an air-pouch. After 24 h of stabilization, a single dose of 20 mg DMBA in 0.5 ml of olive oil (as uniform suspension) was injected into the air pouch.

Five experimental groups having ten animals in each group were used for the study. The normal control group (Group I) administered with 0.5 ml olive oil in air pouch, the induced control group (Group II) animals were treated with DMBA (20 mg in 0.5 ml olive oil in air pouch) only, DAP treatment groups (Group III, IV, and V) were treated with 20 mg/kg, 40 mg/kg and 80 mg/kg b.w. (i.p.) doses respectively along with the DMBA-induction as that of group II. Tumour location was checked and monitored by palpation each week after the induction process. After the promotional stage (90 days after the DMBA-induction), the animals were treated with DAP in different doses. After 28 days of treatment with DAP, blood samples were collected from all groups of experimental animals by retro-orbital puncture and serum was separated for biochemical assessments. Later animals were sacrificed and perfused. Liver, kidney, and breast tissues were isolated and homogenized in phosphate buffer saline (PBS) at 4 °C and used for the biochemical estimations. Fractions of mammary tissues were kept immediately in liquid nitrogen for protein and mRNA preparations using standard protocols. A slice (2–4 mm) of the breast tissue was also processed for the immunofluorescence analysis.

2.3. Effect of DAP on lipid peroxidation and antioxidant marker enzymes

Lipid Peroxidation (LPO) was estimated on the principle of simple TBARS (Thiobarbituric acid reactive species) assay described by Devasagyam [19]. Total superoxide dismutase (SOD), manganese-containing SOD (MnSOD) and copper and zinc-containing SOD (CuZnSOD) activities were determined on the principle of reduction of nitroblue tetrazolium (NBT) [20]. Briefly, the sample aliquots were treated with KCN to deactivate CuZnSOD to estimate MnSOD [21]. CuZnSOD was estimated by calculating the difference between totalSOD and MnSOD. Based on the principle that H_2O_2 reduces dichromate in acetic acid to chromic acetate, catalase (CAT) activity in the test samples was determined [22]. Glutathione peroxidase (GPx) determination in the samples was carried out based on the method described by Rotruck [23]. Protein content was estimated by the method of Lowry [24]. Reduced glutathione (GSH) was estimated by the principle and method described by Ellman [25].

2.4. Assessment of TNF-α and IL-6 by ELISA

The level of specific pro-inflammatory cytokines such as TNF-α and IL-6 were estimated using respective ELISA kits (Biosource, Camarillo, CA) in breast tissue homogenate of control and treated group of animals.

2.5. Effect of DAP on Keap1, Nrf-2 and HO-1 mRNA expression through qRT-PCR

Total RNA was extracted from the mammary tissue (tissues in liquid nitrogen) of all group of animal cells by TRI reagent® (Sigma-Aldrich, India) and purified by adding a mixture containing 150 μL DDW, 8 μL glycogen, 20 μL sodium acetate (3 M), 600 μL of absolute ethanol. The RNA mixture was kept in −80 °C for 2 h and centrifuged at 13000 rpm for 15 min at 4 °C to obtain the purified RNA for cDNA synthesis. According to the manufacturer's instructions, first-stranded cDNA was synthesized by cDNA Synthesis Kit (Bio-Rad) with one μg of total RNA sample and oligo dTTP. Equal amounts of cDNA were subsequently amplified in