Food and Chemical Toxicology 99 (2017) 182–189

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Enhanced chemoprevention by the combined treatment of pterostilbene and lupeol in B[a]P-induced mouse skin tumorigenesis

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ARTICLE INFO

Article history:
Received 21 June 2016
Received in revised form
2 November 2016
Accepted 7 November 2016
Available online 9 November 2016

Keywords:
Pterostilbene
Lupeol
Benzo[a]pyrene
Chemoprevention
Phytochemical

ABSTRACT

The present study is aimed to evaluate the inhibitory effect of the combination of two phytochemicals; pterostilbeneand lupeol (generally obtained from blue berries, grapes, white cabbage, green pepper, olive and mangoes) on mouse skin tumorigenesis. We hypothesized that the concomitant topical treatment of selected phytochemicals would lead to improved impediment of skin cancer. Swiss albino mice (n = 25) received a topical dose of Benzo[a]pyrene (B[a]P, 5 μg/animal) with pre/post application of pterostilbene (16 μM/0.2 ml acetone/animal) and/or lupeol (500 μM/0.2 ml acetone/animal) for 32 weeks. Results showed that pterostilbene and/or lupeol treatment resulted in a significant delay in onset of tumorigenesis. However, a more promising effect on tumor suppression was noted with the combination of both the phytochemicals. A significant reduction in average tumor volume, cumulative number of tumors and tumor multiplicity was recorded in combination treated group. The histopathological analysis illustrated the marked suppression in epidermal hyperplasia and necrotic cells in combination treated groups. Our study suggests that the combination of pterostilbene and lupeol was more effective in prevention of skin cancer as compared to either of the phytochemical alone. Therefore, the combined treatment of phytochemicals has better potential to prevent skin carcinogenesis.

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1. Introduction

For the past few decades, the sustained efforts have been made to utilize the immense potential of natural substances (derivatives/compounds) as preventive agents against many dreadful diseases including cancer (Panahi et al., 2016; Russo et al., 2010; Sirerol et al., 2016). Numerous reports have shown that phytochemicals (biological active components of plants) were able to reduce the prevalence of several types of cancers (Afrin et al., 2016; D'Incalci et al., 2005; Knobloch et al., 2016; Surh, 2003). However, at clinical level, only a limited number of trials were successful (Hosseini and Ghorbani, 2015). Phytochemicals, allium sativum, curcumin, green tea, panax ginseng are among the few agents that showed promising effects, however bioavailability and associated toxicity of these phytochemicals remains a hurdle in translating them at clinical level (Morbidelli, 2016; Nussbaumer et al., 2011). The stratagem of using combined treatment of phytochemicals has demonstrated its potential to inhibit or delay carcinogenesis (Liu et al., 2015; Montgomery et al., 2016). The combined action of two anticancer drugs shows sometimes synergistic preventive effects thereby increases the efficiency of chemoprevention (Arzuman et al., 2016; Kumar et al., 2016).

The dimethyl ether variant of resveratrol, pterostilbene found in some types of berries (Douillet-Breuil et al., 1999; Rimando et al., 2004) have been acknowledged and employed in various studies due to its prospective role towards cancer chemoprevention (Alosi et al., 2010; Douillet-Breuil et al., 1999; Mannal et al., 2010; Pan et al., 2007, 2009; Paul et al., 2009, 2010; Rimando et al., 2004; Schneider et al., 2010). Pterostilbene is superior as compared to other resveraterol-derived compounds in terms of bioavailability and lengthened half-life (Kapetanovic et al., 2011; Remsberg et al., 2008).

Pterostilbene in combination with tamoxifen displayed additive impact in restraining proliferation of breast cancer cells *in vitro* (Mannal et al., 2010). Likewise, pterostilbene in combination with resveratrol displayed synergistic interaction (CI < 1) in inhibition of TNBC cell proliferation. Pterostilbene successfully inhibits Protein kinase B and Bcl-2 cell proliferating factors and induce

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http://dx.doi.org/10.1016/j.fct.2016.11.007
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mitochondrial apoptosis (Chakraborty et al., 2010). Lupeol present in cabbage, mango and olive have shown its anti-cancer properties in a number of *in vitro* and *in vivo* studies (Liu et al., 2015; Pitchai et al., 2014) Prior study from our laboratory has shown that topically applied lupeol avert 7, 12-dimethylbenz[a]anthracene induced DNA strand breaks in murine skin (Nigam et al., 2007). Previously, it has also been demonstrated that lupeol has a preventive action against B[a]P evoked mouse bone marrow cells clastogenicity (Prasad et al., 2008a).

In spite of mounting evidences in support of cancer preventive role of both these phytochemicals, the combinatorial effect of pterostilbene and lupeol has not been studied so far. Henceforth, we have undertaken the present study to estimate the chemopreventive effectiveness of pterostilbene and lupeol combinations in prevention of murine skin tumorigenesis.

2. Material and methods

2.1. Reagents

Pterostilbene, Lupeol, propidium iodide (PI) and dichlorodihydrofluoresceindiacetate dye (DCFH-DA) were purchased from Sigma Chemical Co. (St. Louis, Columbia). Proliferating cell nuclear antigen (PCNA) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Substrate chromogen system diaminobenzidine was procured from Dako, CA, USA. The rabbit anti-mouse or goat anti-rabbit horse radish peroxidase (HRP) conjugate secondary antibodies were procured from Bangalore Genei (Bangalore, India). The remaining chemicals were of analytical grade of purity and procured locally.

2.2. Animals and treatment

Male Swiss albino mice (25 ± 2 g body weight) were obtained from the Indian Institute of Toxicology Research (Lucknow, India) animal-breeding colony following ethical approval for the experiment from institutional ethical committee (IITR/IAEC/32/2014). Mice were kept under standard condition (25 ± 2 °C, relative humidity 57% ± 2% and 12 h:12 h light: dark phase) and fed with synthetic pellet diet (Ashirwad, Chandigharh, India) and water ad libitum. Skin tumors were generated by topical application of B[a]P. The treatment schedule of 5 μg/200 μl B[a]P, thrice in a week was followed according to the procedure of Nigam et al. (2010). The study period was 32 weeks. Animals were divided into differing groups comprising 25 animals in each group as given below:

Group I: Untreated control.

Group II: Vehicle control (Animals received topical treatment of acetone served as vehicle control group (0.2 ml acetone/animal, 3 times in a week).

Group III: Positive control (Animals received topical treatment of B[a]P in acetone served as positive control group (5μg/animal, 3 times in a week)).

Group IV: Animals received topical treatment of pterostilbene alone in acetone (16 μM/0.2 ml acetone/animal, 3 times in a week).

Group V: Animals received topical treatment of pupeol alone in acetone (500 μM/0.2 ml acetone/animal, 3 times in a week).

Group VI: Animals received topical treatment of pterostilbene 30 min prior to B[a]P treatment.

Group VII: Animals received topical treatment of pterostilbene 30 min post to B[a]P treatment.

Group VIII: Animals received topical treatment of lupeol 30 min prior to B[a]P treatment.

Group IX: Animals received topical treatment of Lupeol 30 min post to B[a]P treatment.

Group X: Animals received combinatorial topical treatment of Pterostilbene along with Lupeol 30 min prior to B[a]P treatment.

Group XI: Animals received combinatorial topical treatment of Pterostilbene along with Lupeol 30 min post to B[a]P treatment.

Treatments were given thrice a week for 32 weeks.

Animals from all the groups were examined for gross morphological changes locally on skin and tumor development throughout the experiment period. Average tumor volume was calculated using the formula:

$$V = D \times d2\pi/6,$$

where 'D' is the biggest dimension of the tumor and'd' is the smallest dimension of the tumor.

Cumulative number of tumors was counted weekly. At the end of the study, the animals were sacrificed. The skin from the painted area was excised, washed in chilled phosphate-buffered saline (PBS), and the fat layer was removed using a sterilized scalpel blade. The skin tissues were stored at−80 °C (New Brunswick Scientific Germany) until used for further experimentation.

2.3. Histopathological analysis

After the treatment schedule animals were sacrificed and the skin lesions were harvested and fixed in 10% buffered formalin solution. Fixed tissues were processed using automatic tissue processor (Microm International, STP-120) and paraffin tissue blocks were prepared using tissue embedding system (TES99, MediteGmbh, Germany). Blocks were cut with rotary microtome (Leica RM2055) resulting in 4–5 nm thick sections of the tissues. Slides were stained by routine H & E staining techniques as per SOP/HTP/001. Slides were observed under optical microscope (Leica, Wetzler, Germany).

2.4. Immunohistochemical (IHC) staining

Harvested skin lesions/tissues was fixed in 10% buffered formalin, transferred formalin, transferred to cassates following multiple baths of increasing concentrations of ethanol cleared using xylene, and finally embedded in paraffin. Tissues were cut into 5 mm thick sections. The endogenous peroxidase activity was quenched and epitope retrieval was done followed by blocking of non-specific binding of primary antibody to epitopes by a pre-incubation step with normal serum. Overnight incubation of the sections was done with primary monoclonal anti-PCNA (1:100) antibody. Later, the sections were incubated with respective HRP-conjugated secondary antibody. The color was developed by substrate chromogen system diaminobenzidine (Dako, CA, USA). The immunostained slides were examined under microscope (Leica, Wetzler, Germany) attached with charge coupled device camera (JVC).

2.5. Flow cytometry analysis of cell cycle

Cell cycle analysis was done by flow cytometry technique. Skin tissue/tumors were extracted from treated and control groups for preparing single cell suspensions using Medimachine (Beckton Dickinson, San Jose, USA). The single cell suspensions were fixed in chilled ethanol (70%). The cells were centrifuged from the fixative followed by Triton X-100 (0.1%) treatment for 5 min. Thereafter, cells were washed in PBS and resuspend in 1 ml of PBS and 50 μg/ml PI with 200 μg/ml Ribonuclease. The cells were incubated for 30 min in dark at room temperature. The samples were acquired by using Flow cytometer and analyzed by using 'Cell Quest software'.
2.6 Measurement of ROS levels.

To determine ROS generation using DCFH-DA dye through flow

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