Cancer Chemother Pharmacol (2010) 65:687–696
DOI 10.1007/s00280-009-1074-x

ORIGINAL ARTICLE

Induction of apoptosis by [6]-gingerol associated with the modulation of p53 and involvement of mitochondrial signaling pathway in B[a]P-induced mouse skin tumorigenesis

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Received: 14 March 2009 / Accepted: 8 July 2009 / Published online: 24 July 2009

Abstract
Purpose To unravel the molecular mechanisms underlying the chemopreventive potential of [6]-gingerol, a pungent ingredient of ginger rhizome (*Zingiber officinale Roscoe*, *Zingiberaceae*), against benzo[a]pyrene (B[a]P)-induced mouse skin tumorigenesis.
Methods Topical treatment of [6]-gingerol (2.5 μM/animal) was given to the animals 30 min prior and post to B[a]P (5 μg/animal) for 32 weeks. At the end of the study period, the skin tumors/tissues were dissected out and examined histopathologically. Flow cytometry was employed for cell cycle analysis. Further immunohistochemical localization of p53 and regulation of related apoptogenic proteins were determined by Western blotting.
Results Chemopreventive properties of [6]-gingerol were reflected by delay in onset of tumorigenesis, reduced cumulative number of tumors, and reduction in tumor volume. Cell cycle analysis revealed that the appearance of sub-G1 peak was significantly elevated in [6]-gingerol treated animals with post treatment showing higher efficacy in preventing tumorigenesis induced by B[a]P. Moreover, elevated apoptotic propensity was observed in tumor tissues than the corresponding non-tumor tissues. Western blot analysis also showed the same pattern of chemoprevention with [6]-gingerol treatment increasing the B[a]P suppressed p53 levels, also evident by immunohistochemistry, and Bax while decreasing the expression of Bcl-2 and Survivin. Further, [6]-gingerol treatment resulted in release of Cytochrome *c*, Caspases activation, increase in apoptotic protease-activating factor-1 (Apaf-1) as mechanism of apoptosis induction.
Conclusions On the basis of the results we conclude that [6]-gingerol possesses apoptotic potential in mouse skin tumors as mechanism of chemoprevention hence deserves further investigation.

Keywords Mouse skin tumorigenesis · [6]-Gingerol · Benzo(a)pyrene · Chemoprevention · Apoptosis

Introduction

Developing novel strategies to prevent skin cancer represents an urgent goal due to increasing rise in incidence of skin cancer patients throughout the world [1, 2]. Not surprisingly, more than one million new cases of basal cell and squamous cell carcinoma are diagnosed in the United States every year, accounting for 40% of all cancer cases [3, 4]. In Asian population, the incidence of skin cancer is comparatively less. India is one of the low incidence regions of the world subjected to skin cancer. Cancer registries in India report that the age specific incidence rates for skin cancer are less than 0.5 per 1,000,000 [5]. However, because skin lesions are visible and easily accessible, skin cancers provide us with an excellent in vivo model to study the development of cancers [6].

Naturally occurring phytochemicals present an active cancer preventive strategy to inhibit, delay, or reverse human carcinogenesis. Studies have indicated that certain daily-consumed dietary phytochemicals have cancer protective effects mediated by carcinogens. Ginger, the powdered rhizome of the herb *Zingiber officinale*, is widely

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used as a traditional treatment in the Ayurvedic medicine, where it has been used as a treatment for various chronic diseases [7]. The main pungent compounds in fresh ginger are a series of homologous phenolic ketones known as gingerols, the major being [6]-gingerol. [6]-Gingerol has been shown to suppress promotion of skin carcinogenesis in laboratory animals [8, 9]. Park et al. [9] reported that [6]-gingerol inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA) skin tumor promotion in addition to the inhibition of tumor necrosis factor-alpha production, epidermal ornithine decarboxylase activity in ICR mice. Another study revealed that topical application of the ethanol extract of ginger resulted in suppression of TPA mediated induction of ornithine decarboxylase and its mRNA expression in SENCAR mouse skin. Pre-application of ginger extract to mouse skin afforded significant inhibition of TPA caused epidermal edema (56%) and hyperplasia (44%) [8]. Topical application of [6]-gingerol inhibited TPA-induced cyclooxygenase-2 (COX-2) expression along with suppressed nucler factor-kappa B (NF-κB) DNA binding activity in mouse skin [10]. Recently, topical application of [6]-gingerol (30 μM) prior to ultra-violet B irradiation (5 kJ/m^2) of hairless mice, has been reported to inhibit the induction of COX-2 mRNA and protein level, as well as NF-κB translocation and by blocking the p38 MAP kinase-NF-κB signaling pathway [11]. Based on the previous studies on chemopreventive properties of [6]-gingerol, we examined the apoptotic potential of [6]-gingerol on benzo[a]pyrene (B[a]P)-induced skin tumorigenesis assay by targeting mitochondrial pathway of apoptosis as mechanism of cancer chemoprevention. This study also set a bracket of chemopreventive potential of [6]-gingerol in tumor tissues versus the corresponding non-tumor tissues on [6]-gingerol treatment. Therefore, suggesting the beneficial effects of [6]-gingerol and its potential to be developed as a potent non-toxic agent against skin cancer.

Materials and methods

Chemicals

[6]-Gingerol, B[a]P, β-actin and propidium iodide (PI) were purchased from Sigma Chemical Company (St Louis, CA, USA). Bcl-2 (Ab-2) and Bax (Ab-1) rabbit polyclonal IgG, antibodies were procured from Oncogene Research Products (Cambridge, USA). Survivin, Caspase 3, Caspase 9, Cytochrome *c*, Poly (ADP-ribose) polymerase (PARP), apoptotic protease-activating factor-1 (Apaf-1), p53 antibodies was procured from Cell Signaling Technology (Beverly, MA, USA). The rabbit anti mouse or goat anti rabbit horseradish peroxidase conjugate secondary antibodies were obtained from Bangalore Genei (Bangalore, India). The nitrocellulose membranes were obtained from Millipore (Bedford, MA, USA). The rest of the chemicals were of analytical grade of purity and were procured locally.

Animal bioassay

In order to determine the strength or biological activity of [6]-gingerol, mouse skin carcinogenesis model was used. For this, Swiss albino mice (male, 20–22 g body weight) were obtained from Indian Institute of Toxicology Research (Lucknow, India) animal breeding colony. The ethical approval for the experiment was obtained from institutional ethical committee. The animals were caged in polypropylene cages and housed 15 animals per cage on wood chip bedding in an air-conditioned (temperature 23 ± 2°C, relative humidity 55 ± 5%) animal room. Animals were quarantined for 1 week on a 12/12 h light/dark cycle and were fed solid pellet diet (Ashirwad, Chandigarh, India) and water *ab libitum*. The mouse skin tumors were obtained by using B[a]P (5 μg/animal) as a complete carcinogen as described earlier [12]. In brief, B[a]P and/or [6]-gingerol (2.5 μM/animal) were applied topically on shaved dorsal skin in the interscapular region of 2 cm^2. The dose was selected on the basis of literature review [13]. The animals were divided into 5 groups comprising 15 animals each. Briefly, animals of Gr. I (untreated control) were kept without any treatments. Gr. II animals were only applied [6]-gingerol topically in acetone (200 μl). Animals of Gr. III were applied B[a]P in acetone (200 μl) topically, which served as a positive control. In order to study the chemopreventive effects of [6]-gingerol, animals of Grs. IV and V were topically applied [6]-gingerol, 30 min prior and post to B[a]P, respectively. Treatments were given thrice a week for 32 weeks. Animals from all the Grs. were examined throughout the experiment for gross morphological changes locally on skin, including loss of hair and development of tumors. Average tumor volume was calculated using the formula $V = D \times d^2\ \pi/6$, where 'D' is the biggest and 'd' is the smallest dimensions of the tumor. Cumulative number of tumors (CNT) was also counted to evaluate the effect of [6]-gingerol on tumor development. Further the animals of [6]-gingerol treated Grs. IV and V, were divided into two sub Grs. prior to sacrifice i.e., Grs. IVA and VA comprising of tumor-bearing animals and Grs. IVB and VB comprising of animals which had no tumor induction during the entire study period. All the animals were then sacrificed after 24 h of the last treatment at the end of the study period. Skin from the 2 × 2 cm^2 interscapular region (with or without tumors) was excised, cleaned, and snap frozen in liquid nitrogen, and stored at −80°C (New Brunswick Scientific, Germany) for further analysis.

Histopathological analysis

Six micrometer thick sections of the tissues were cut using cryostat microtome (Slee, Mainz, Germany) and were processed as per as standard procedure, stained with haematoxylin and eosin and examined under a microscope (Leica, Wetzler, Germany). Histopathological classification of the tumors was performed as described by Bogovaski [14].

Immunohistochemical localization of p53

Immunohistochemistry was performed as described by Arora et al. [15] with slight modifications. For this, frozen skin/tumor sections (10 μm) were cut using cryostat microtome (Slee, Mainz, Germany) and were fixed in neutral 10% phosphate-buffered formaldehyde solution. The endogenous peroxidase activity was quenched with methanol and 30% hydrogen peroxide (H_2O_2) solution (9:1) and the non-specific binding was blocked with 1% normal goat serum in Tris buffer saline. The slides were sequentially incubated with anti-p53 antibody in a moist chamber for 72 h at 4°C. After incubation, the sections were again incubated with normal goat serum and then with horseradish peroxidase–conjugated antigoat IgG secondary antibody. The slides were washed with phosphate buffer saline (PBS) between incubations. The color was developed using chromagen 3-methyl 9-ethyl carbazole in Tris (0.05 mol/L, pH 7.3) buffer containing H_2O_2. For all the studies samples from all Grs. were analyzed individually.

Image analysis

The immunostained slides were analyzed under microscope attached with CCD camera (JVC, Tokyo, Japan) and Leica QWin 500 image analysis software for each slide in triplicates with at least six microscopic fields.

Flow cytometric analysis of cell cycle

The single cell suspensions of skin tissue/tumors from different treated and control Grs. were prepared using Medimachine (Beckton Dickinson, San Jose, USA) as described earlier [16]. For the cell cycle analysis, cells in suspension were fixed in chilled 70% ethanol. Cells were centrifuged from the fixative and treated with 0.1% Triton X-100 for 5 min. After incubation, cells were washed in PBS and resuspended in 1 ml of PBS and 50 μg/ml PI with 100 μg/ml Ribonuclease A followed by incubation for 30 min in dark at room temperature. The samples were acquired and analyzed on flow cell cytometer (Becton-Dickinson LSR II, San Jose, CA, USA) using 'Cell Quest' software.

Preparation of lysates

For lysates preparation, fat was scrapped off from skin/tumor tissues, on ice individually. The samples were then homogenized in ice-cold lysis buffer (50 mM Tris–HCl, 150 nM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP-40, 1% Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, pH 7.4), which were then placed over ice for 30 min [17]. The lysates were collected in microcentrifuge tubes and passed through 21G needle to break up the cell aggregates. The lysates were cleared by centrifugation at 14,000*g* for 15 min at 4°C and the supernatant was either used immediately or stored at −80°C.

Western blotting

Western blotting was carried out as described earlier [15]. Protein concentration was estimated by the method of Lowry et al. [18] using bovine serum albumin as standard. Briefly, proteins (100 μg) were resolved on 5–10% sodium dodecyl sulphate-polyacrylamide gels and electroblotted on nitocellulose membranes. The blots were blocked overnight with 5% non-fat dry milk and probed with various monoclonal and polyclonal antibodies at dilutions recommended by the suppliers. Immunoblots were detected through chemiluminescence using enhanced chemiluminescence reagents obtained from Millipore (Billerica, MA, USA). Data is presented as the relative pixel density of the protein bands normalized to β-actin. The intensities of the bands were quantitated by using UN-SCAN IT software (Orem, UT, USA).

Statistical analysis

The data was analyzed for mean values and standard error (SE) for all treated and untreated control Grs. which were subjected to statistical comparison using student *t* test by using SPSS-12 software, $p < 0.05$ was considered as significant. Survival analysis of animals was carried out by Kaplan–Meier curve using SYSTAT 9.0 software.

Results

Effect of [6]-gingerol on B[a]P-induced mouse skin tumorigenesis

The results showed the chemopreventive activity of [6]-gingerol on B[a]P-induced mouse skin tumorigenesis. Skin lesions were observed both in experimental and positive control Grs. The lesions were confined to the interscapular region of the skin and they were broadly classified as

Table 1 Effect of [6]-gingerol on B[a]P- induced mouse skin tumorigenesis

Groups (Grs.)	Treatment	I induction of tumor (in days)	Number of animals with tumors	% of animals with tumors	Cumulative no. of tumors (CNT)	Avg. no. of tumors/ tumor bearing mouse (Mean ± SE)	Avg. tumor vol./ tumor bearing mouse (in $mm^{3)}$ (Mean ± SE)
I	Untreated control	–	0/15	0	–	–	–
II	[6]-gingerol	–	0/15	0	–	–	–
III	B[a]P	161st	15/15	100**	128**	8.53 ± 1.47**	87.6 ± 12.2 **
IV	[6]-gingerol + B[a]P	199th	11/15	73*	58*	5.27 ± 0.95*	44.9 ± 8.6 *
V	B[a]P + [6]-gingerol	206th	9/15	60*	34*	3.78 ± 0.76*	31.0 ± 4.9 *

** Significant ($p < 0.05$) increase over control Gr. I. * Significant ($p < 0.05$) decrease over B[a]P treated Gr. III. Details of treatment are provided in "Materials and methods"

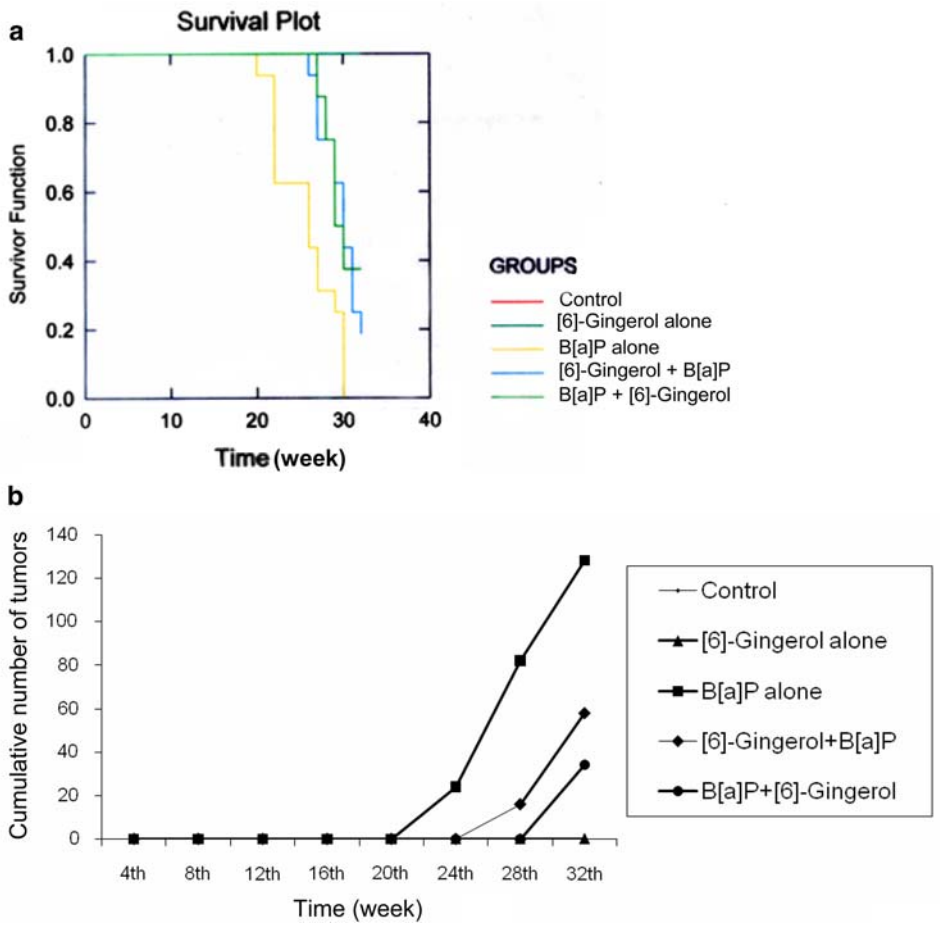

Fig. 1 **a** Kaplan–Meier curve for the determination of tumor free survival by [6]-gingerol treatment on B[a]P induced tumorigenesis. The *vertical axis* shows the percentage of tumor free survival and the *horizontal axis* shows the weeks of treatment. **b** Effect of [6]-gingerol on incidence of tumorigenesis in terms of cumulative number of tumors

tumors and non-tumors lesions. The non-tumor lesions included loss of hair followed by poor hair growth at the site of application, hyperkeratinization and scaly skin.

The animal bioassay revealed a delay in the onset of tumorigenesis in [6]-gingerol treated Grs. as compared to the Gr. treated with B[a]P alone. The induction of first tumor was observed on day 161 in B[a]P treated animals (Gr. III) but the onset of tumorigenesis was observed on 199th and 206th day in [6]-gingerol treated Grs. IV and V respectively (Table 1). The onset of tumorigenesis was delayed by around 5 weeks in [6]-gingerol treated animals in Grs. IV and V in comparison to animals treated with B[a]P only, i.e. Gr. III (Table 1). The chemopreventive potential of [6]-gingerol was also evident by increase in tumor free survival of animals (Fig. 1a). The anti-tumorigenic effects of [6]-gingerol in B[a]P-induced tumorigenesis were also observed when calculated in terms of the percentage of animals with tumors. Protection could also be seen in terms of reduction in tumor volume. The tumor volume was 87.6 ± 12.2 mm^3 per animal in B[a]P treated

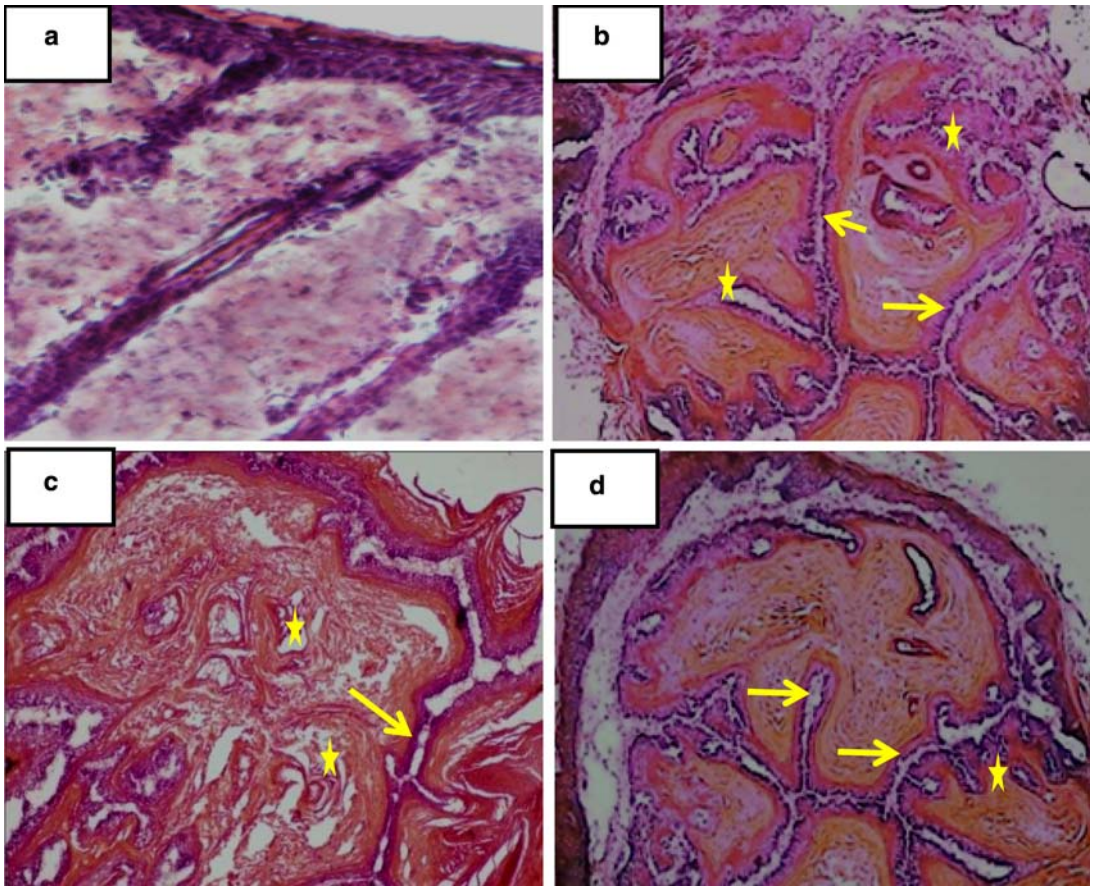

Fig. 2 Histopathology of mouse skin sections stained with haematoxylin and eosin showing **a** untreated control mouse skin section showing normal thickness of the epithelium (20×), **b** skin tumor section that developed by B[a]P treatment (Gr. III) showing disorganized squamous epithelial cells with high nuclear and cytoplasmic ratio (20×), **c** skin tumor section of Gr. VI, in which [6]-gingerol was administered prior to B[a]P treatment, showing changes towards normalization of skin as compared to B[a]P treated Gr. III (20×), **d** skin tumor section of Gr. V, in which [6]-gingerol was administered post to B[a]P treatment, showing changes towards normalization of skin as compared to both Grs. III and VI (20×)

Gr. II, but it was only 44.9 ± 8.6 mm^3 and 31.0 ± 4.9 mm^3 in animals of [6]-gingerol treated Grs. IV and V, respectively. Thus, [6]-gingerol treatment resulted in 51% (Gr. IV) and 65% (Gr. V) suppression in tumors volume. The chemopreventive effect of [6]-gingerol was also evident in terms of reduction in CNT and average number of tumors per tumor bearing mouse. The CNT in Gr. III was 128 at the termination of the experiment while, it was 58 and 34 in Grs. IV and V, respectively (Fig. 1b). Thus, [6]-gingerol treatment resulted in 55% (Gr. IV) and 74% (Gr. V) reduction in tumor induction induced by B[a]P (Table 1). Similarly, topical treatment of [6]-gingerol resulted in 5.27 ± 0.95 and 3.78 ± 0.76 tumors/tumor bearing mouse in Grs. IV and V, respectively in comparison to B[a]P treated Gr. with 8.53 ± 1.47 tumors/tumor bearing mouse which was a significant decrease ($p < 0.05$) (Table 1). Histopathological examination and p53 immunohistochemical staining were performed to visualize the influence of [6]-gingerol treatment on the aggressiveness of tumors induced by B[a]P. For further experiments, we analyzed the matched tissue pairs of tumor bearing and non-tumor bearing animals in the [6]-gingerol treated Grs. with the [6]-gingerol alone treated and positive control at cellular and protein levels.

Histopathological examination

Histologically, the B[a]P treated Gr. III (Fig. 2b) exhibited varying degrees of structural and cytological divergence in skin sections as compared to untreated control Gr. I (Fig. 2a) and [6]-gingerol treated Gr. II (data not shown) in our experimental sets. Skin section of animals belonging to B[a]P treated Gr. showed changes in basement membrane and squamous cell hyperplasia at few places. Tumors of animals belonging to B[a]P treated experimental Grs. were composed of focal proliferation of squamous cells (arrow), presence of some necrotic cells (asterisk), and occasional presence of epithelial pearls. This clearly indicates the malignant nature of the tumors caused by B[a]P as a complete carcinogen. Figure 2c, d shows that topical application of [6]-gingerol leads to suppression of

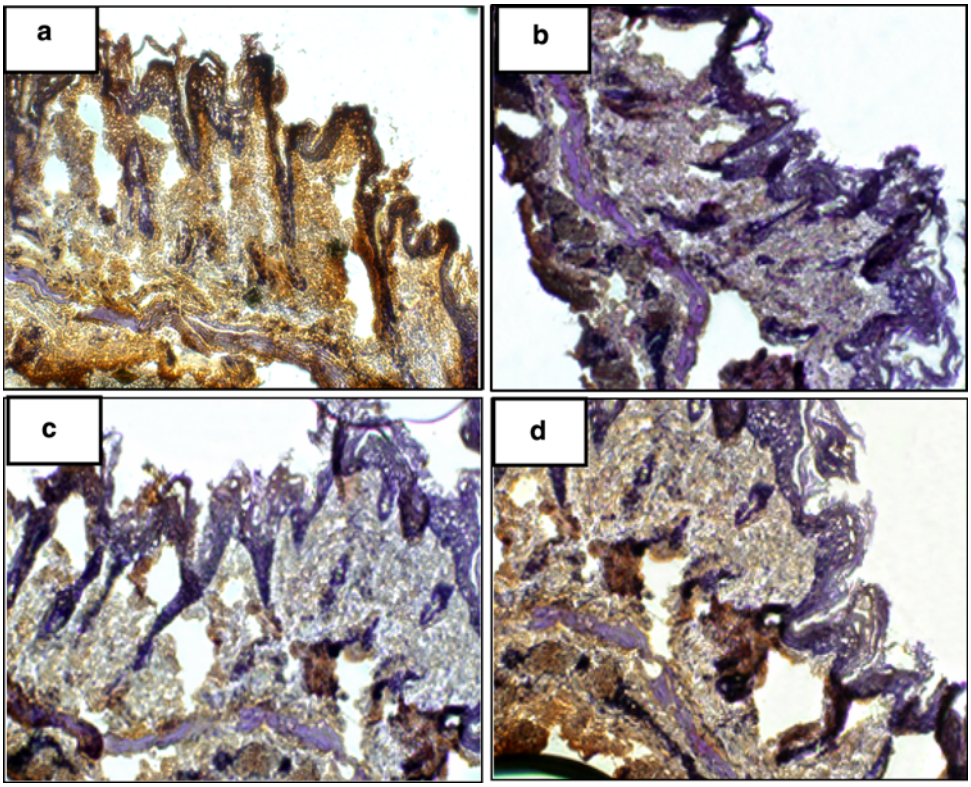

Fig. 3 Modulatory effect of [6]-gingerol application on p53 expression in different Grs. Details are described in "Materials and methods". [6]-Gingerol application leads to increased expression of p53 expression in B[a]P-induced tumors. The brown deposits of 3-methyl 9-ethyl carbazole mark the reactivity with p53. **a** Untreated control, **b** B[a]P alone, **c** [6]-gingerol + B[a]P, **d** B[a]P + [6]-gingerol (10×)

skin tumorigenesis in mice of Grs. IV and V. However, Gr. IV, in which treatment of [6]-gingerol was given 30 min prior to B[a]P (Fig. 2c), less pronounced preventive effects were noticed as compare to Gr. V animals, in which treatment of [6]-gingerol was given 30 min post to B[a]P (Fig. 2d).

Effect of [6]-gingerol on tumor suppressor protein, p53

p53 in response to toxic insults to DNA, triggers a chain of cell cycle regulatory events to check the proliferation of altered cells to repair or minimize the damage [19, 20]. A characteristic intense staining and higher number of positive cells were observed and indicated by arrows in Fig. 3. In tumors, loss of p53 function prevents the activation of this growth control pathway via cell cycle control and apoptosis [21]. It was observed that the number of p53 positive cells were lower in animals treated with B[a]P alone (Gr. III, Fig. 3b) as compared to untreated control animals (Gr. I, Fig. 3a). The expression of p53 in animals of [6]-gingerol alone (Gr. II) was comparable to untreated control (Gr.I) (data not shown). Treatment with [6]-gingerol prior and post to B[a]P treated animals resulted in appreciably higher levels of p53 positive cells (Grs. IV and V) (Fig. 3c, d).

Effect of [6]-gingerol on induction of apoptosis

In the present study, we observed that [6]-gingerol acts on cell cycle regulation through the appearance of sub-G1 peak indicative of apoptosis confirming its apoptotic potential in B[a]P applied mouse skin. [6]-Gingerol induced accumulation of cells in the sub-G1 phase was observed largely in Gr. V as compared to Gr. IV with concomitant decrease in G1 phase against B[a]P-mediated carcinogenic events. Moreover, analyzing the matched tissue pairs of tumor bearing and non-tumor bearing animals, [6]-gingerol -pretreated Grs. IVA and IVB corresponded to 33.7 and 5.5% apoptosis, while, [6]-gingerol post-treated Grs. VA and VB corresponded to 45.3 and 14.3% apoptosis, respectively, over 0.9% in [6]-gingerol alone treated and 1.5% in untreated controls (Fig. 4).

Effect of [6]-gingerol on Bax and Bcl-2 protein

Consistent with immunohistochemical analysis, Western blot analysis revealed that topical application of [6]-gingerol could effectively modulate the expression levels of p53 in comparison to B[a]P exposed mouse skin. We observed a marked decrease in expression of p53 in B[a]P exposed mouse skin (Gr. III) over control (Gr. I). However, [6]-gingerol treatment up-regulated the expression of p53 as observed largely in Gr. V as compared to Gr. IV with higher influence on Grs. IVA and VA than the non-tumor bearing animals of Grs IVB and VB. No significant ($p < 0.05$) difference in the expression levels of p53 was observed between the control Grs. I and II (Fig. 5a).

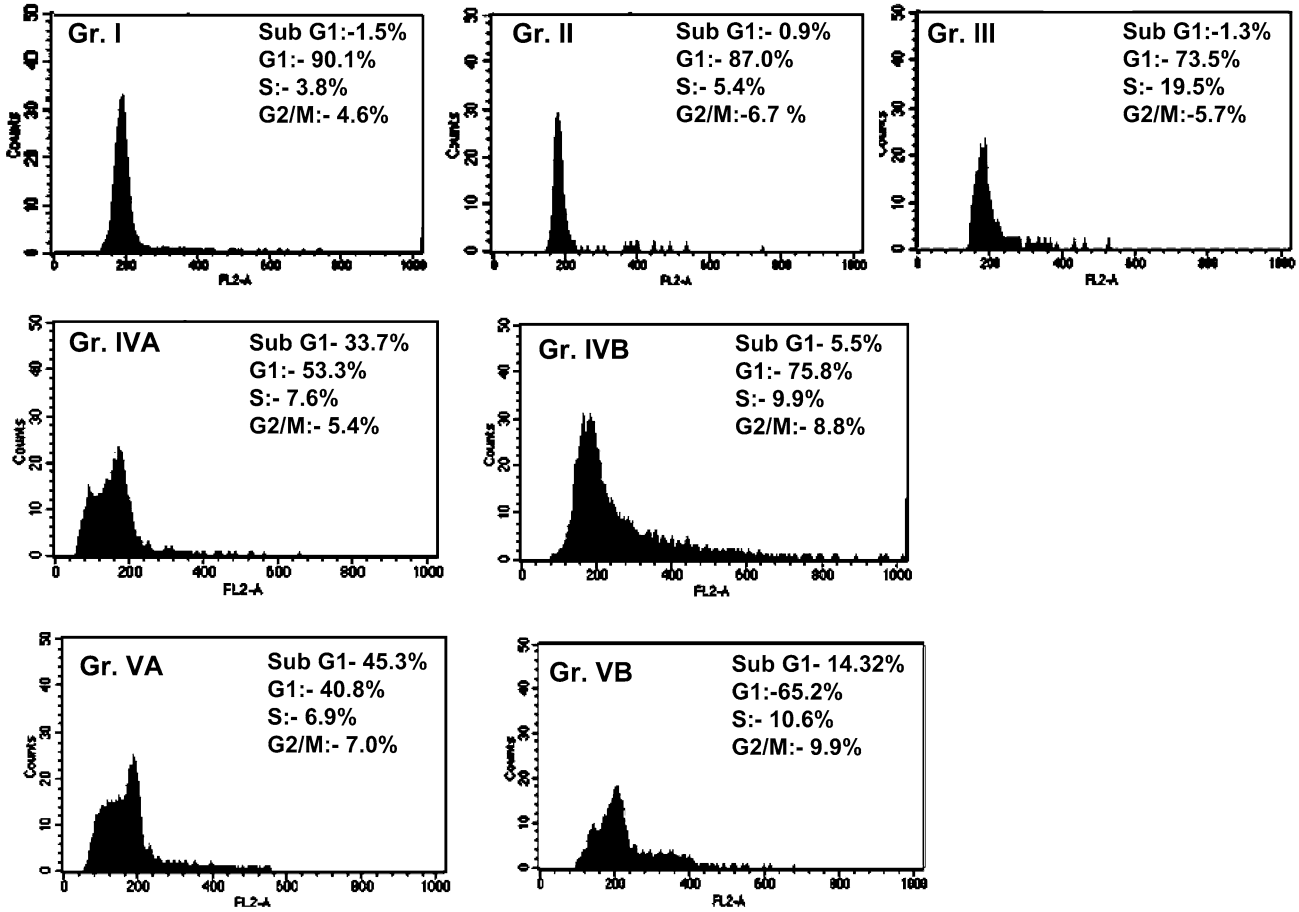

Fig. 4 Cell cycle analysis histograms of apoptosis in mouse skin in different Grs. PI fluorescence was measured using a flow cytometer with FL-2 filter and were expressed as histogram

Mitochondrial permeability leads to the release of apoptogenic factors which is promoted by Bax, a downstream regulator of p53 while Bcl-2 inhibits its effect [22]. We further, ascertained the modulating effect of [6]-gingerol on Bcl-2 family proteins. Bax was down-regulated in B[a]P treated Gr. III while its expression was elevated in [6]-gingerol treated Grs. IV and V observed largely in Gr. V as compared to Gr. IV with higher influence on Grs. IVA and VA than the non-tumor bearing animals of Grs IVB and VB (Fig. 5b). Conversely, Bcl-2 and Survivin were up-regulated in B[a]P treated animals (Gr. III). However, the B[a]P-induced expression of Bcl-2 and Survivin were down-regulated in [6]-gingerol treated animals (Grs. IVA/B and VA/B) (Fig. 5c, d) in a fashion opposite to that of p53 and Bax.

Effect of [6]-gingerol on Cytochrome *c* release, Caspase activation and PARP cleavage

Mitochondrial permeability leads to release of apoptogenic factors like Cytochrome *c*, Apaf-1 and activation of Caspase 9, Caspase 3, and PARP cleavage. Release of Cytochrome *c* into cytosol and expression of Apaf-1 were down-regulated in B[a]P treated animals over control Gr. I. [6]-Gingerol treatment elevated level of cytosolic Cytochrome c (Fig. 5e) and expression level of Apaf-1 (Fig. 5f) in comparison to B[a]P treated Gr. III. However, results of Western blotting also showed cleavage of Caspase-9 (35 and 37 kDa), Caspase-3 (19 and 17 kDa) and PARP (116, 85, and 62 kDa) on [6]-gingerol treatment (Fig. 6a–c). Similar trend of expression levels was observed in all these proteins with higher apoptotic inclination observed in Gr. V as compared to Gr. IV. This was pronounced in the tumor bearing animals of Grs. IVA and VA than in the non-tumor bearing animals of Grs IVB and VB.

Discussion

It is being increasingly appreciated that chemoprevention with natural agents may be a plausible strategy for the management of skin cancer [2]. Studies have shown chemopreventive properties of [6]-gingerol against skin cancer [23, 24]. In the present investigations, the anti-tumor activity

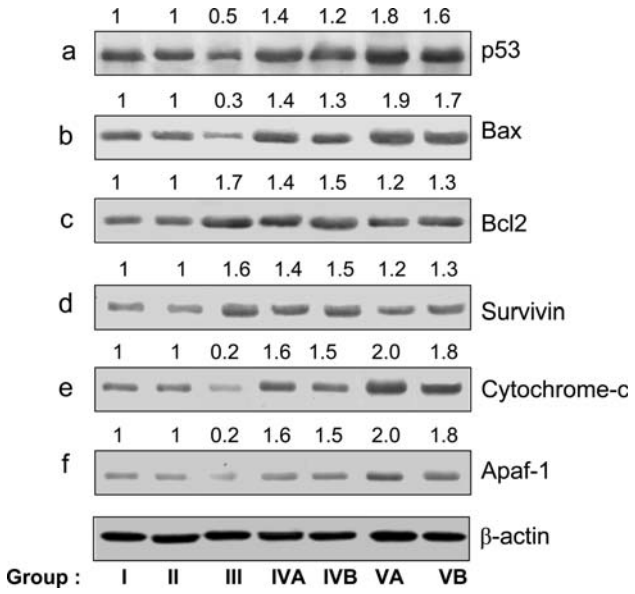

Fig. 5 Western blots showing the effect of [6]-gingerol on **a** p53, **b** Bax, **c** Bcl-2, **d** Survivin, **e** Apaf-1, **f** Cytochrome *c*, in mouse skin/tumors in different Grs. Gr. I: untreated control, Gr. II: [6]-gingerol alone, Gr. III: B[a]P alone, Gr. IVA: [6]-gingerol + B[a]P with tumors, Gr. IVB: [6]-gingerol + B[a]P without tumors, Gr. VA: B[a]P + [6]-gingerol with tumors, Gr. VB: B[a]P + [6]-gingerol without tumors. The *bands* shown here are from a representative experiment repeated three times with similar results and expressed as mean of their relative pixel density normalized to *β*-actin

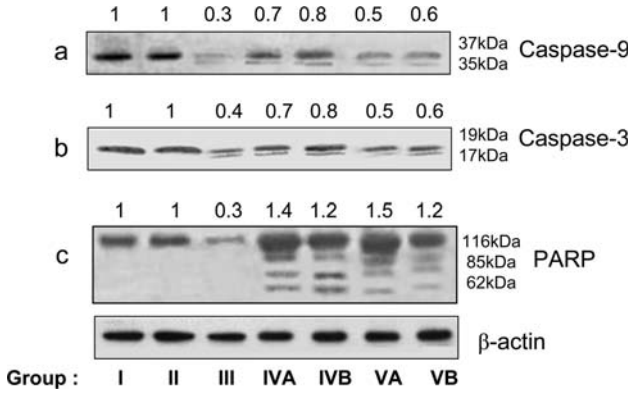

Fig. 6 Western blots showing the effect of [6]-gingerol on **a** Caspase 9, **b** Caspase 3, **c** PARP in mouse skin/tumors in different Grs. Gr. I: untreated control, Gr. II: [6]-gingerol alone, Gr. III: B[a]P alone, Gr. IVA: [6]-gingerol + B[a]P with tumors, Gr. IVB: [6]-gingerol + B[a]P without tumors, Gr. VA: B[a]P + [6]-gingerol with tumors, Gr. VB: B[a]P + [6]-gingerol without tumors. The *bands* shown here are from a representative experiment repeated three times with similar results and expressed as mean of their relative pixel density normalized to *β*-actin

of [6]-gingerol was observed in mouse skin model of carcinogenesis (Table 1).

The animal bioassay revealed a significant delay in onset of tumorigenesis, significantly reduced CNT and reduction in tumor volume. Hence, overall tumorigenesis study clearly showed a strong protective effect of [6]-gingerol treated post to B[a]P when compared to prior treatment against B[a]P- induced mouse skin tumorigenesis. Results of cell cycle analysis clearly demonstrated that [6]-gingerol treatment post to B[a]P application induced apoptosis more significantly as compared to prior treatment (Fig. 4). The apoptotic tendency was also pronounced in tumor tissues than the corresponding non-tumor tissues. Lee et al. [25] recently reported that [6]-Gingerol treatment suppressed cell proliferation, caused G(1) cell cycle arrest and induceed apoptosis followed by modulation of cyclin D1 expression. In another study, Yagihashi et al. [26] reported the anti-oxidative property of [6]-gingerol leading to the induction of apoptosis via cell cycle arrest together with anti-invasive activity in cancer cells.

We further extended this work to gain insight into the signaling network and interaction points modulated by [6]-gingerol via ascertaining their role in modulation of the proteins involved in the mitochondrial pathway of apoptosis. The ability of p53 to eliminate excess, damaged or infected cells by apoptosis is vital for the proper regulation of cell proliferation in multi-cellular organisms [27]. p53 participates in apoptosis induction by acting directly at mitochondria. Localization of p53 to the mitochondria occurs in response to apoptotic signals and precedes Cytochrome *c* release and Caspase-3 activation. Recently, Mihara et al [28] also extended this finding to show that p53 promotes permeabilization of the outer mitochondrial membrane by forming complexes with the protective Bcl-XL and Bcl-2 proteins thereby down-regulating these anti-apoptotic proteins [29]. In our study, we found that the expression of p53 and its downstream regulator Bax was increased and Bcl-2 was decreased on [6]-gingerol treatment. Increased expression of Bax can induce apoptosis by suppressing the activity of Bcl-2 [30, 31], confirming that the ratio of Bcl-2 and Bax is crucial for the apoptosis induced by chemopreventive agents [32]. Interaction among the Bcl-2 family proteins (Bax, Bak, Bcl-2, Bcl-X, etc) stimulates the release of Cytochrome *c* which promotes the formation of apoptosome with Apaf1 which in turn activates executioner Caspases to orchestrate apoptosis. Caspases are the crucial components of the apoptosis pathway. The important step in activation of the cell death program is the activation of Caspase 3 and Caspase 9 [33]. PARP is a protein involved in a number of cellular processes involving mainly DNA repair and programmed cell death [34]. Consistent with the above studies, our study also showed the up-regulation of the proteins like Cytochrome C, Apaf1, Caspase 9, Caspase 3 and PARP in [6]-gingerol supplemented Grs. in comparison to B[a]P treated Gr. The ability of PARP is to repair damaged DNA which is prevented through its cleavage by executioner Caspases [35]. Survivin

protein, member of inhibitor of apoptosis protein family, directly inhibits apoptosis and its expression is found to be frequently high in cancer cells and correlated with resistance to chemotherapy [36, 37]. Survivin helps in invasion of apoptosis, possibly by impairing Caspases activation and mitochondrial dysfunction [36]. Similar observation was found in our study, showing increased level of Survivin in B[a]P exposed Gr. However, [6]-gingerol treatment modulated the Survivin protein and inducing apoptosis probably through activation of Caspases. Similar trend of expression levels was observed in all these proteins with higher apoptotic inclination observed in post [6]-gingerol treatment with higher influence on the tumor bearing animals than the non-tumor bearing animals.

These observations confirm that [6]-gingerol acts as a suppressing agent against skin carcinogenesis. We also conclude through our results that apoptotic potential of [6]-gingerol acts in tumor tissues is higher than the corresponding non-tumor tissues and can regulate intrinsic apoptotic pathways by directly triggering apoptosis-promoting signaling cascades as mechanism of cancer chemoprevention. It is hopeful that further characterization of pathways regulating cell cycle progression and apoptosis will facilitate novel drug discovery programs to exploit [6]-gingerol for the prevention and treatment of several human cancers.

Acknowledgments Authors are thankful to Director Indian Institute of Toxicology Research, Lucknow (Council of Scientific & Industrial Research, India) for his keen interest in the study. We are thankful to Dr. Neeraj Mathur, Scientist for statistically analyzing the data. Authors are also thankful to Department of Biotechnology (India) and Council of Scientific & Industrial Research, New Delhi (under NWP-17) for jointly funding this work.

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