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Antiangiogenic Effects of Synthetic Analogs of Curcumin *in vivo*

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ABSTRACT

The active compound curcumin is isolated from the spice turmeric. Curcumin, curcuminoids and their synthetic analogs have been shown to inhibit the progression of cancer in animal models. In colon and skin carcinogenesis the genetic changes engross different genes, but curcumin is effective in preventing carcinogenesis in both organs. A needful elucidation for this result is that analogues of curcumin can inhibit angiogenesis. The synthetic analog of Curcumin, 1,5 bis (3,5 dimethoxy phenyl) 1,4 pentadiene –3 one (BDMP) was tested for its capacity to inhibit the proliferation of primary endothelial cells in the presence and absence of the basic fibroblast growth factor (bFGF) and its ability to inhibit proliferation of an immortalized endothelial cell line. BDMP and other analogs of curcumin such as bis (3,4 dimethoxyphenyl) 1,3 propanedione (BDMPP), bis (2,4 dimethoxyphenyl) 1,3 propanedione (DMPP), and bis (3,3 dinitrophenyl) 2-bromo 1,3 propanedione (BDNP) were subsequently tested for their ability to inhibit bFGF-induced corneal neovascularization in the mouse cornea. Ultimately, BDMP was evaluated for its ability to inhibit phorbol ester-stimulated vascular endothelial growth factor (VEGF) mRNA production. BDMP effectively inhibited endothelial cell proliferation in a dose dependent manner. BDMP, BDMPP, DMPP and BDNP have shown significant inhibition of bFGF mediated corneal neovascularization in the mouse. BDMP had no effect on phorbol ester stimulated VEGF production. Results of these investigations show that BDMP has direct antiangiogenic activity *in vitro* and *in vivo*. Carcinogenesis inhibiting activity of the BDMP in skin and colon may be mediated in part through angiogenesis inhibition.

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Key words: - Angiogenesis, Curcumin analogs, BDMP, Neovascularization

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INTRODUCTION

Spice turmeric is a foremost constituent of the diet of the Indian subcontinent. A carotenoid pigment curcumin purified from the rhizome of *curcuma longa*, source of turmeric (Ammon and Wahl, 1991, Stoner and Mukhtar, 1995). Mice treated with skin and colon carcinogens were shown decreased size of tumors on treatment with curcumin compared to control mice (Huang *et al.*, 1994, Rao *et al.*, 1995, Conney *et al.*, 1991, Huang *et al.*, 1995, Huang *et al.*, 1992). Inhibition of several signal transduction pathways has been verified by curcumin, including those involving arachidonic acid metabolisms, protein kinase C, phospholipase A₂ bioactivity, the transcription factor NF-κB, antioxidant activity, and epidermal growth factor (EGF) receptor autophosphorylation (Lu *et al.*, 1994, Singh and aggarwal, 1995, Huang *et al.*, 1991, . Korutal *et al.*, 1995, Rao *et al.*, 1993)

In westernized nations skin and colon cancers are major public health problems. 20% of colon carcinoma in the United States due to hereditary colon cancer. Colon carcinogenesis in human disease and transgenic mice is the contribution of some genes (Dietrich *et al.*, 1993, Su *et al.*, 1992). These syndromes are usually autosomal dominant and they include ancestral adenomatous polyposis and non-polyposis syndromes. In mice phospholipase A₂ expression has been verified to be a co-factor in genetically vulnerable mice. (Macphee *et al* 1995) In addition, deletion of the cyclooxygenase2 gene in colon carcinoma – prone Apc mice results in suppression of intestinal polyposis (Oshima *et al.*, 1996). Loss of P⁵³ and activation of *ras* oncogenes appear later in tumor progression (Hinds *et al.*, 1990, Redston *et al.* 1995). Human skin cancers comprise the most general form of human neoplasia and are growing in frequency, principally as a result of sun exposure. In compare to colon carcinoma mutations of the p53 gene are felt to arise early in ultraviolet induced cutaneous carcinogenesis. Curcumin and its analogues have caring effects against skin and colon cancer, but the genes concerned in tumor development of skin and colon cancer differ, we hypothesized that the anti tumor

effects of the BDMP may be due in part to angiogenesis embarrassment. At this point we reveal that BDMP inhibits basic fibroblast growth factor (bFGF) induced proliferation of endothelial cells *in vitro* and angiogenesis *in vivo*. In addition tested the effect of BDMPP, DMPP and BDNP on *in vivo* angiogenesis. Our results specify that inhibition of angiogenesis may trigger in part the antitumor activity of BDMP *in vivo*.

MATERIALS AND METHODS

Curcumin analogs BDMP, BDMPP, DMPP, BDNP were commercially purchased from Sigma (USA), Dulbecco's modified Eagle's medium from Hi- media Mumbai (India), 12-O-tetradecanoyl phorbol-13- acetate, VEGF, Basic fibroblast growth factor were from (Sigma Aldrich, USA), sucralfate from Bukh Meditec Vaerlose, (Denmark). HaCat keratinocytes cell line purchased from NCCS (Pune India). All other reagents used were of analytical grade.

Animals: Swiss Wister albino male rats weighing 160-200 gm were obtained from Central Animal House facility, Department of Studies in Zoology, University of Mysore, Karnataka, India. Food was given ad libitum before starvation. Animal experiments were approved by the central animals house department of studies in zoology; the animal care and handling were conducted in accordance with CPCSEA office and ophthalmology guidelines University of Mysore division Mysore –India.

Endothelial proliferation assays: Bovine capillary endothelial cells were isolated according to the method of Folkman *et al* (1979) and were plated at a concentration of 10,000 cells/well in gelatinized 24-well dishes. The primary endothelial cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine serum and grown at 37⁰ C in 10 % CO₂. Twenty- four hours after plating, cells were treated with BDMP in the presence or absence of bFGF. After 72 hours of treatment, cells were counted using a coulter counter. Cells

counts for each condition were repeated in triplicate and in the presence or absence on 1 ng/ml bFGF. Similarly, MS1 (ATCC CCRL 2279) endothelial cells, which are a SV 40 large T antigen immortalized murine endothelial cell line (Arbiser *et al.*, 1997) were also plated at a concentration of 10,000 cells / well in non-gelatinized 24-well dishes. MS1 cells not require endothelial mitogens for growth and were cultured in DMEM supplemented with 5% fetal calf serum. Cells were counted after 72 hr exposure to BDMP with the same method used for the bovine capillary endothelial cells.

Evaluation of b FGF induced corneal neovascularization by curcumin analogues:

Pellets were prepared according to a modification of the method of Kenyon *et al.*, 1996. An aqueous solution of 80 ng of basic fibroblast growth factor was evaporated to dryness under reduced pressure in the presence of 10 mg of sucralfate. Ten micro liters of 12 % hydron and 10 mg of BDMP or BDMPP, DMPP, BDNP were then added, and the homogenous mixture was deposited on to a sterile 15x15 mm 3-300/50 nylon mesh and air dried. Once the mixture was dry, the mesh was manually dissociated to yield 225 pellets. Each pellet contained 80 ng of b FGF and 44 µg of BDMP, BDMPP, DMPP and BDNP. Pellets prepared in the absence of b FGF were not used in this study. The approximate pore size was 0.4x0.4 mm. Both sides of the mesh were covered with a thin layer of hydron. Male Swiss Wistar rats were anesthetized with methoxyflurane prior to implantation of pellets and with 0.5% proparacaine. A central, intrastromal linear keratotomy was performed with a surgical blade, and a lamellar micropocket was prepared. The pellet was advanced to the end of the pocket. Erythromycin ointment was placed on the operated eye to prevent infection. Eyes were examined by slit lamp on day 3-6 after implantation under general anesthesia. Corneal angiogenesis was assayed through two measurements. The first measurement, vessel length, is the length of the vessels from the corneal limbus as it grows towards the bFGF pellet. A clock hour is a measurement of neovascularized area of the

cornea. The cornea is viewed as a circle that can be divided as a clock, with a maximum of 12 hrs. Thus a measurement of 3 clock hours implies that one quarter of the cornea is vascularized. This system of measurement was established by Kenyon *et al* (1996).

RNase protection for VEGF: HaCat keratinocytes (Boukamp *et al.*, 1998) were grown in (DMEM) supplemented with 55 FCS in 25 cm² flasks. One hour prior to stimulation with 12-O-tetradecanoyl phorbol-13- acetate (TPA), cells were switched to serum less media supplemented with 10 µM DMP or an equal quantity of ethanol (final concentration 0.1%). TPA was added to a final concentration of 5ng/ml and incubated for 3 hr at 37°C. Cells were harvested and RNA extracted with guanidinium thiocyanate/phenol. A plasmid containing the coding region of human vascular endothelial growth factor (VEGF) 121 used to generate P³²- labeled antisense riboprobe as per manufacturer protocols (Ambion, Austin, TX). RNase protection assays were performed according to the method of Hod (1992) Protected fragments were separated on gels of 5% acrylamide, 8M urea, 1X Tris-borate buffer, and quantified with a phosphor imager. An 18 S riboprobe was included in each sample to normalize for variations in loading and recovery of RNA.

Phase II Enzyme Induction: The ability of curcumin derivatives to induce phase II activities was measured by assaying quinone reductase [NAD (P) H :(quinone –acceptor) oxidoreductase, EC 1.6.99.2] in murine Hepa2c7 cells. Serial dilutions of BDMP, BDMPP, DMPP, and BDNP were added, and the concentration of compound required to double the specific activity (CD) was calculated according to the method of Prochaska *et al.*, (1992).

Statistics

Significant differences between two groups were determined using an unpaired, two tailed student's t-test. Results are expressed as the mean ± standard error of the mean.

RESULTS

Inhibitory effect of BDMP on Endothelial proliferation in the presence or absence of bFGF: Endothelial cells are stimulated to proliferate in the presence of 1ng/ml bFGF. BDMP was added in concentration ranging from 0.5-10 μ M to primary endothelial cells. A steep decrease in cell number was seen at 10 μ M. No evidence of cytotoxicity was observed, and the number of cells at the end of was not significantly less than the number of cells originally plated. This decrease was observed in either the presence or absence of bFGF (Fig 1). In addition, BDMP was able to inhibit the growth of endothelial cells immortalized by SV40 large T antigen, with a similar dose response as seen with primary endothelial cells.

BDMP inhibit b FGF-induced Neovascularization in the mouse cornea: The capacity of BDMP to inhibit b FGF –induced corneal neovascularization in vivo was studied. Pellets were prepared containing 80 ng of b FGF and BDMP or a control aromatic ketone, tetraphenylcyclopentadienone (TPCPD). TPCPD was added to rule out the possibility that the inhibition of neovascularization due to BDMP was not secondary to dilution. There was no difference in neovascularization in mice containing b FGF pellets in the presence or absence of TPCPD. Neovascularization was assessed by slit lamp at 5 days after implantation, and the corneas were photographed. Both the vessel length and clock hours were significantly reduced in the presence of BDMP (Fig2).

Saturated Analogs of Curcumin Inhibit b FGF-Induced Neovascularization in the Mouse cornea: analogs of curcumin were assayed for their ability to inhibit bFGF induced corneal neovascularization as described above. All analogs showed inhibitory activity, with BDMPP showing the greatest activity on both clock hours and vessel length, BDNP having the least effect on clock hours, and DMPP having the least effect on vessel length (Fig3). All of the curcumin derivatives showed significant inhibition of bFGF mediated neovascularization compared with control pellets.

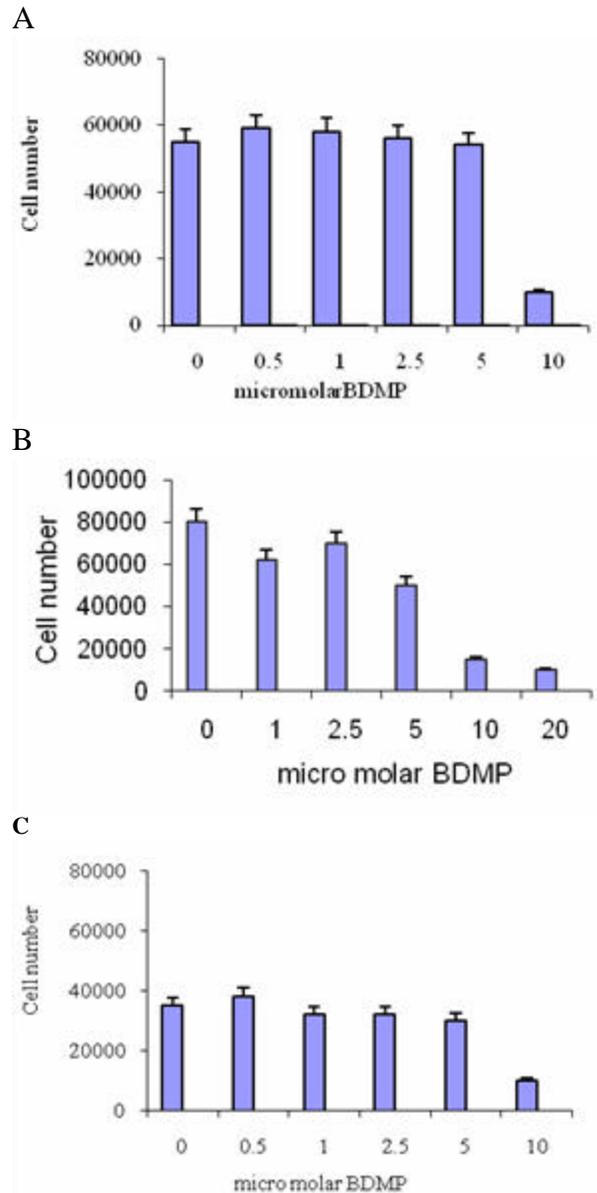


Fig 1: Effect of BDMP on the proliferation of capillary endothelial cells. Cells were grown in the presence or absence of b FGF and doses of BDMP ranging from 0 to 20 μ M. The y-axis denotes the cell number after 72 hours. (A) Effect of BDMP on primary capillary endothelial cells stimulated with 1 ng/ml of bFGF. (B) Effect of BDMP on the proliferation of unstimulated primary capillary endothelial cells. (C) Effect of BDMP on immortalized endothelial cells. The error bars represent standard error of the mean

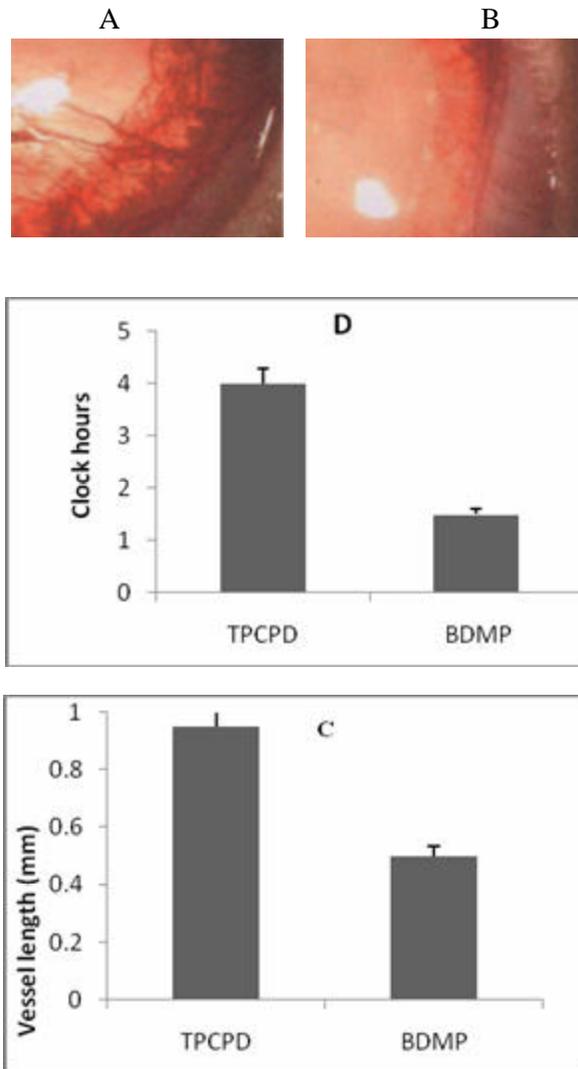


Fig.2: Effect of BDMP on corneal neovascularization. (A, B) The photograph on the left shows a cornea containing bFGF and BDMP, while the cornea on the right contains TPCPD, a control substance, and an equal quantity (80ng) of bFGF. Corneal vessel enlarged in the absence of BDMP and in attenuated in the presence of BDMP. (C) The bar graph shows the effect of BDMP and TPCPD on vessel length. $P < 0.05$. (D) The bar graph shows the effect of BDMP and TPCPD on clock hours, a measure of area. The error bars represent standard error of the mean.

Effect of Curcumin Derivatives on Induction of phase II enzyme Induction: To determine whether the antiangiogenic activities of the curcumin derivatives correlated with the ability to induce quinone reductase activity. The CD

(concentration to double the specific activity) value for BDMP was $6.3 \mu\text{M}$, $7.0 \mu\text{M}$ for BDMPP and $10.0 \mu\text{M}$ for DMPP. These CD values are approximately equal, but they differ significantly from that of tetrahydrocurcumin. Tetrahydrocurcumin, the curcumin derivative with the least anti tumor activity, caused a 1.2 fold induction of quinone reductase activity at $25 \mu\text{M}$. However, TPCPD, which is an unsaturated aromatic ketone with no anti-angiogenic activity, had a CD value of $4.8 \mu\text{M}$. Thus anti-angiogenic activity does not correlate with phase II activity.

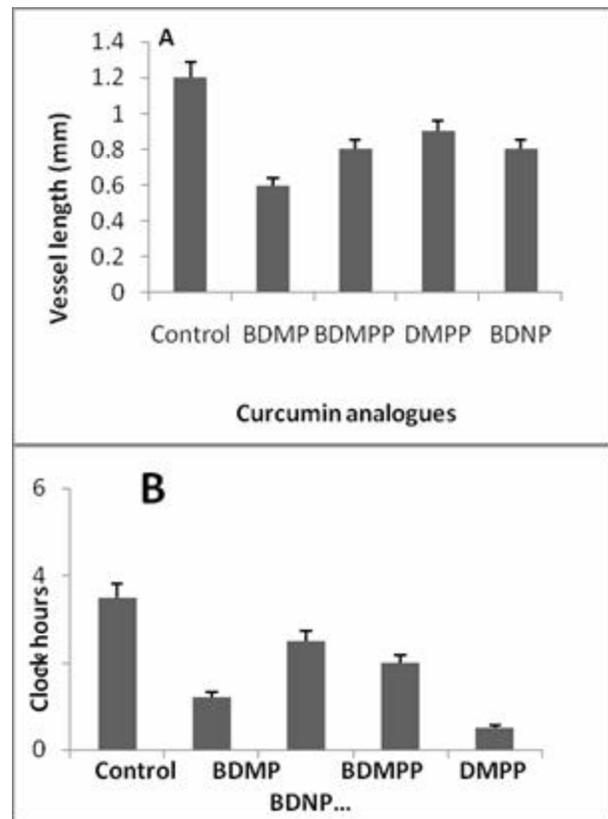


Fig.3: Effect of curcumin analogues on corneal neovascularization. (A) Effect of curcumin analogs on vessel length. All curcumin analogs show a significant difference in vessel length from controls at $p < 0.05$. (B) Effect of curcumin analogs on clock hours. All curcumin analogs show a significant difference in area from controls at $p < 0.05$.

Effect of BDMP on VEGF production by transformed keratinocytes: Ha Cat cells are

derived from spontaneously transformed human keratinocytes (Boukamp *et al.*, 1988). In order to determine whether BDMP could inhibit production of angiogenesis factors by relevant tumor cells as well as directly inhibit endothelial function, we treated Ha Cat cells with tetradecanoylphorbol ester (TPA) in the presence or absence of BDMP and assayed expression of VEGF mRNA. TPA caused a 2.5-fold increase in VEGF mRNA, which was not inhibited by BDMP (Fig4). Thus the primary anti-angiogenic effect of BDMP is directly on endothelium, rather than inhibition of production of VEGF, an important angiogenic factor.

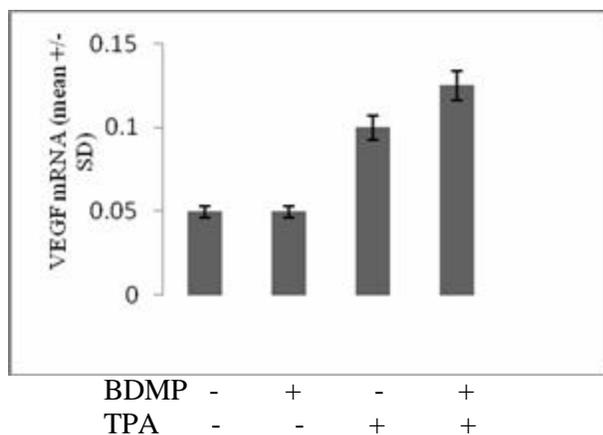


Fig.4: Effect of BDMP on VEGF mRNA production in Ha Cat cells. The intensity of bands from VEGF RNase protection were quantified by densitometry and normalized for loading by quantification of 18S RNA. The error bars represent standard error of the mean.

DISCUSSION

Curcumin is a harmless compound with well-known chemo preventive activity in areas of direct contact, such as the gastrointestinal tract and skin (Huang *et al.*, 1994, Rao *et al.*, 1995, Conney *et al.*, 1991, Huang *et al.*, 1995, Huang *et al.*, 1992). The reasons for its effectiveness as a chemo preventive agent are not fully stated. Curcumin has been shown to have several activities that may affect tumorigenesis *in vivo*. Earliest, curcumin has been shown to inhibit tumor beginning by the carcinogens benzopyrene and 7,12, - dimethylbenzanthracene (DMBA), as well as tumor beginning induced by phorbol esters (Huang

et al., 1995, Huang *et al.*, 1992, Rao *et al.*, 1993). Curcumin has also been shown to inhibit phorbol ester induced ornithine decarboxylase, a marker of cellular proliferation and tumorigenesis, in mouse skin. In addition, curcumin has an inhibitory effect on expression of *c-fos* and *c-jun*, oncogenes that form the transcription factor AP-1. (Lu *et al.*, 1994, Huang *et al.*, 1991). Moreover these pathways influence tumor cell proliferation *in vivo*, and curcumin may have a dual effect on inhibiting both tumor growth *in vivo* through inhibiting tumor progression, as well as inhibiting angiogenesis in tumors that have already undergone the angiogenic switch.

The corneal neovascularization assay, which measure vessel length and density in response to a bFGF pellet placed in the normally avascular cornea, has confirmed useful in the characterization of multiple angiogenesis inhibitors (Kenyon *et al.*, 1996) Potent angiogenic factor is basic fibroblast growth factor (bFGF). This aspect has been shown to be an effective stimulus for both endothelial proliferation and migration. The action of b FGF on endothelial cells may be due in part though stimulation of protein kinase C (Kent *et al.*, 1995). Administration of BDMP or BDMPP, DMPP, BDNP within the pellet resulted in powerful inhibition of bFGF-induced corneal neovascularization. This inhibition was not due to dilution of bFGF, as administration of a structurally related inactive compound, tetraphenylcyclopentadienone (TPCPD), has no effect on bFGF- induced corneal neovascularization.

In the presence or absence of bFGF, BDMP had a potent antiproliferative effect on the endothelial cells, with a steep curve occurring between 5 and 10 μ M, and this inhibition could not be overcome by the immortalizing ability of SV 40 large T antigen.

According to earlier reports, curcumin, demethoxycurcumin, bis- demethoxycurcumin were able to inhibit phorbol ester-stimulated induction of ornithine decarboxylase and promotion of mouse skin initiated with 7, 12-dimethylbenzanthracene (DMBA) (Huang *et al.*, 1995). These derivatives also inhibited phorbol

mediated ester-mediated transformation of JB6 cells (Lu *et al.*, 1994) Synthetic saturated and unsaturated analogues of curcumin were placed into corneal pellet and assayed for their angiogenesis inhibition. BDMP, BDMPP, DMPP, BDNP were all active in inhibiting bFGF induced neovascularization, but to unreliable degrees. BDMP, BDMPP, DMPP are to a great extent chemo preventive agent than BDNP, although all of the derivatives have antiangiogenic activity. This may be due to other activities of BDMP, such as the ability to induce phase II detoxifying enzymes, which may inhibit further tumor promotion. Unsaturated derivative BDMP has potencies in induction of phase II enzymes, where as the fully saturated BDNP has slight ability to induce phase II enzymes.

Two types of angiogenesis inhibitors have been classified such as direct angiogenesis inhibitors, which are relatively specific for endothelial cells and have little effect on tumor cells (Arbiser *et al.*, 1997). These comprise soluble VEGF receptor antagonists and angiostatin (Kim *et al.*, 1993, O'Reilly *et al.*, 1994). Indirect inhibitors may not have direct effects on endothelial cells but may down regulate the making of an angiogenesis stimulator, such as VEGF. (Arbiser *et al.*, 1997, Gess *et al.*, 1996). VEGF has been shown to be up regulated during chemically induced skin carcinogenesis; this is likely due to activation of oncogenes, such as H-ras (Arbiser *et al.*, 1997, Larcher *et al.*, 1996, Kohl *et al.*, 1995) Indirect inhibitors of angiogenesis comprise inhibitors of *ras* mediated signal transduction, such as farnesyltransferase inhibitors (Kohl *et al.*, 1995) The antagonism of bFGF-mediated corneal neovascularization by BDMP and BBMP, BDMPP, DMPP, BDNP propose that BDMP is a direct inhibitor of angiogenesis.

Anticancer agents are diverse group of compounds with various activities. We confirm that BDMP, another chemopreventive agent, is able to inhibiting bFGF-mediated angiogenesis in vivo. The exact mechanism of how chemopreventive agents actually prevent neoplasia is not fully understood. Angiogenesis inhibition may trigger in part the valuable activity of cancer

preventive agents.

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