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Inhibition of Tumor Specific Angiogenesis by Amentoflavone

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Abstract—The formation of new capillaries from existing blood vessels is critical for tumor growth and metastasis. In this study we report that amentoflavone, a biflavonoid from *Biophytum sensitivum*, could inhibit the process of angiogenesis. Amentoflavone at nontoxic concentrations (0.05-0.2 μg/ml) showed significant inhibition in the proliferation, migration, and tube formation of endothelial cells, which are key events in the process of angiogenesis. *In vivo* studies in C57BL/6 mice using amentoflavone showed remarkable inhibition (52.9%) of tumor directed capillary formation. Amentoflavone showed inhibitory effect on the production of various endogenous factors such as IL-1β, IL-6, TNF-α, GM-CSF, and VEGF that control the process of angiogenesis. Amentoflavone treatment could increase the production of IL-2 and TIMP-1, which could successfully shift the equilibrium towards an angiostatic condition. The antiangiogenic activity of amentoflavone was supported by its remarkable suppression in sprouting of microvessels from rat aorta. Our results also show that amentoflavone could inhibit the production of VEGF mRNA in B16-F10 cells. These findings indicate that amentoflavone inhibits angiogenesis by disrupting the integrity of endothelial cells and by altering the endogenous factors that are required for the process of neovascularization.

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Key words: amentoflavone, angiogenesis, endothelial cells, proinflammatory cytokines, tube formation

Angiogenesis, which involves the growth of new capillaries from preexisting microvessels, is critical for various physiologic and pathologic processes, particularly tumorigenesis and metastasis. Angiogenesis is a highly regulated process and is essential for embryogenesis. This process is active in adults only during ovulation, cyclical endometrial proliferation, and wound repair [1]. A variety of distinct proangiogenic molecules as well as a number of antiangiogenic effector molecules were associated to modulate neovascularization related to tumor formation and progression [2-5]. These molecules can affect various signaling pathways and stimulate the migration and proliferation of the component cell types and to establish functional blood vessels [6]. It is expected that drugs that block the molecular events responsible for tumor angiogenesis will be effective against a broad spectrum of tumor types. Therefore, inhibitors of angiogenesis are predicted to be better tolerated than conventional cytotoxic cancer therapies that affect all rapidly growing cells.

Flavonoids are ubiquitous polyphenolic compounds found in vascular plants, which are endowed with a large variety of biological effects. Biflavonoids are a unique class of naturally occurring flavonoids. Amentoflavone (Fig. 1), a biflavonoid present in *Biophytum sensitivum*, has been shown to possess antiviral [7, 8], anti-inflammatory [9], antidepressant [10], antioxidant [11], and analgesic [12] activities. Amentoflavone is an inhibitor of phospholipase Cγ1 [13], an irreversible inhibitor of lymphocyte proliferation [14], and an inhibitor of nitric oxide synthase in macrophages [15]. It shows considerable inhibition of cAMP phosphodiesterase in rat adipose tissues [16], inhibits non-enzymic lipid peroxidation [17], and inhibits cathepsin B, a member of papain super family of cysteine proteases [18]. It is a potent scavenger of superoxide [19]. It has shown inhibitory effect on the degradation of IκBα and NF-κB translocation into the nucleus [20].

Abbreviations: bFGF) basic fibroblast growth factor; ELISA) enzyme-linked immunosorbent assay; FCS) fetal calf serum; GM-CSF) granulocyte monocyte colony stimulating factor; HUVEC) human umbilical vein endothelial cell; IL) interleukin; iNOS) inducible nitric oxide synthase; MMP) matrix metalloproteinase; MTT) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TIMP) tissue inhibitor of metalloproteinase; TNF) tumor necrosis factor; VEGF) vascular endothelial growth factor.

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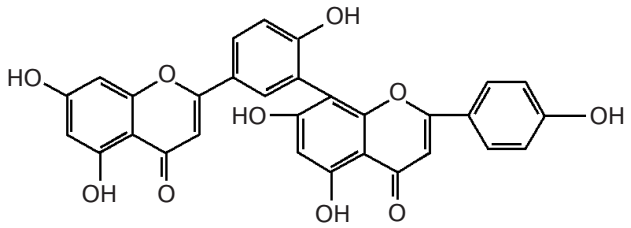

Fig. 1. Chemical structure of amentoflavone.

In the present study, we show that amentoflavone has a profound inhibitory effect on angiogenesis *in vitro* and *in vivo*. Amentoflavone treatment inhibited the proliferation, migration, and tube formation of endothelial cells, which are key events in the process of angiogenesis. Consistent with *in vitro* experiments, *in vivo* experiments using B16-F10 melanoma cells in the C57BL/6 mouse model also showed that amentoflavone significantly suppressed tumor angiogenesis. The *in vivo* antiangiogenic activity correlated with altered cytokine levels, lowered VEGF (vascular endothelial growth factor) mRNA levels, and decreased tumor microvessel formation. Our data indicate the antiangiogenic activity of amentoflavone and may help further improve its effectiveness in controlling cancer growth and metastasis.

MATERIALS AND METHODS

Reagents and chemicals. Amentoflavone was isolated from *Biophytum sensitivum* as previously described [21] and compared with an authentic sample by thin layer chromatography, ultraviolet absorption, ^{13}C-NMR, and mass spectrum. Amentoflavone or TNP-470 (from Dr. Ravivarma, NCI, USA) were solubilized in dimethylsulfoxide (0.1%) or suspended in gum acacia (1%) for *in vitro* or *in vivo* studies, respectively.

ELISA (enzyme-linked immunosorbent assay) kits for mouse IL (interleukin)-1β, IL-6, TNF-α (tumor necrosis factor α), GM-CSF (granulocyte monocyte colony stimulating factor), and IL-2 were purchased from Pierce Endogen (USA). VEGF and TIMP-1 (tissue inhibitor of metalloproteinase 1) ELISA kits were purchased from R & D System (USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (USA). All chemicals used are of reagent or higher grade.

Cell lines and tissue culture. HUVECs (human umbilical vein endothelial cells) were isolated from human umbilical cord veins by collagenase treatment as described previously [22]. HUVECs were maintained in a gelatin-coated 75-cm^2 flask using M199 (HiMedia, India), supplemented with 20% (v/v) fetal calf serum (FCS) (Biological Industries, Israel), 100 units/ml penicillin, 100 μg/ml streptomycin, 500 ng/ml basic fibroblast growth factor (bFGF) (Pepro Tech Inc., USA), and 100 ng/ml VEGF (Pepro Tech Inc). B16-F10 mouse melanoma cells (National Centre for Cell Sciences, Pune, India) were maintained as a monolayer culture in DMEM (HiMedia) supplemented with 10% (v/v) FCS and antibiotics. All the cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Animals. C57BL/6 (5-6-week-old) male mice (20-25 g) were purchased from the National Institute of Nutrition (Hyderabad, India). All mice were housed, cared for, and used in strict accordance with the rules and regulations of the Institutional Animal Ethics Committee, Government of India.

Determination of cell viability by MTT assay. Endothelial cells (10^6/ml) were incubated with increasing concentrations of amentoflavone (0.05-10 μg/ml) for six days in 96-well plates. The cell viability was calculated by measuring the absorbance at 570 nm after incubation with MTT for 4 h at 37°C [23, 24].

***In vitro* angiogenesis assay.** Rat aorta was cultured as previously described with slight modifications [25]. Briefly, male Sprague–Dawley rats weighing 230 g were euthanized by decapitation; their thoracic aorta was rapidly excised and placed in PBS containing 100 units/ml penicillin and 100 μg/ml streptomycin. Adventitia was removed under sterile conditions and the aorta was rinsed three times with PBS and cut into 1 mm rings. The aortic rings were cultured for six days in collagen coated wells along with conditioned medium (100 μl) from B16-F10 cells and increasing concentrations of amentoflavone (0.05-0.2 μg/ml). The newly formed microvessels from aortic ring were counted under a phase contrast microscope and the percentage growth inhibition was calculated.

$$\text{Percentage growth inhibition} = (C - T)/C \cdot 100,$$

where C and T represent control and treated groups, respectively.

Endothelial cell proliferation assay. Endothelial cells ($5 \cdot 10^4$ cells) were cultured for 24 h in the presence of increasing concentrations of amentoflavone (0.05-0.2 μg/ml) along with VEGF (2 ng/ml), and the proliferation of endothelial cells was determined by the [^{3}H]thymidine incorporation assay [26]. The recovered radioactivity was measured using a Rack Beta liquid scintillation counter (Wallac 1209; Pharmacia, Sweden) and expressed as counts per minute (cpm).

Wound migration assay *in vitro*. *In vitro* wound migration assays were carried out as previously described [27]. A single wound was created using a sterile plastic tip in the center of the endothelial cell monolayer. After creating the wound, the cells were cultured for 48 h in the presence of increasing concentrations of amentoflavone (0.05-0.2 μg/ml) along with VEGF (2 ng/ml). The cells were stained for 10 min with 0.1% (w/v) crystal violet, and the

cells migrating to the wounded area were counted and photographed under the phase contrast microscope (40× magnification).

Tube formation assay. Endothelial cell tube formation assay was performed as previously described [28]. Endothelial cells ($5 \cdot 10^4$ cells) were cultured for 48 h in Matrigel (Sigma) coated wells in the presence of increasing concentrations of amentoflavone (0.05-0.2 μg/ml) along with VEGF (2 ng/ml). The cells were fixed with formaldehyde (5%), stained for 10 min with Diff Quick (Dade Behring Inc, USA), and photographed under the phase contrast microscope (40× magnification).

VEGF mRNA quantification. The VEGF mRNA preparations were made from B16-F10 (10^6) cells cultured in the presence of increasing concentrations of amentoflavone (0.05-0.2 μg/ml) according to the manufacturer's (Quantikine, R & D Systems, USA) instructions and their level were quantified spectrophotometrically at 490 nm. Values are expressed in attomol/ml (1 attomol (10^{-8} mol) = 600,000 molecules).

***In vivo* angiogenesis assay.** Male C57BL/6 mice were divided into three groups (six animals per group). Angiogenesis was induced by injecting B16-F10 (10^6) cells (s.c.) on the shaved ventral side of mice and were treated simultaneously (i.p.) with 10 mg amentoflavone per kg body weight or 30 mg TNP-470 per kg body weight for five consecutive days. Control animals received B16-F10 cells alone. Blood samples were drawn from the retro-orbital plexus at two time points (day 1 and day 9) and centrifuged (400*g*, 10 min) to obtain serum. Serum nitrite [29], cytokine (IL-1β, IL-6, TNF-α, GM-CSF, VEGF), and TIMP-1 levels were quantified using ELISA kits as per the manufacturer's instructions. Three separate experiments were each tested in duplicate. After nine days, the mice were euthanized by decapitation, ventral skin was excised, and the tumor directed capillaries were quantified under a dissection microscope (40× magnification).

Statistics. Each experiment was performed independently at least thrice with similar results; one representative experiment was presented. Values are expressed as mean ± standard deviation and analyzed using one-way ANOVA followed by Duncan's Multiple Range Test for comparisons of group means. Unpaired Student's *t*-test was used for statistical analysis, when only two groups are compared. Unless specified otherwise, a p value <0.05 is considered statistically significant.

RESULTS

Cell viability by MTT assay. Amentoflavone at concentrations 0.05, 0.1, and 0.2 μg/ml showed no toxic effects on endothelial cells (Fig. 2), and these concentrations were used for *in vitro* experiments using endothelial cells.

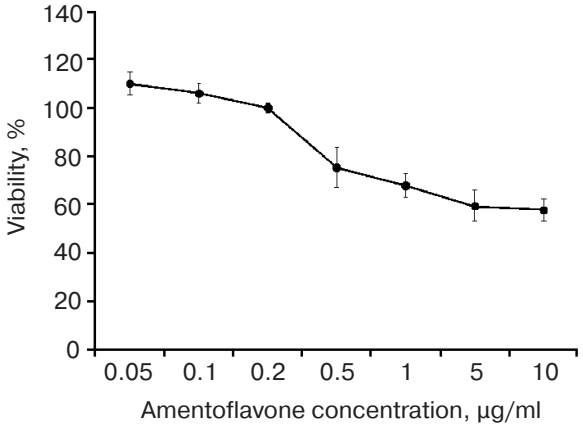

Fig. 2. Effect of amentoflavone on cell growth *in vitro*. Endothelial cells (10^6/ml) were incubated with increasing concentrations of amentoflavone (0.05-0.2 μg/ml) for six days and percent cell viability of each well was measured by MTT assay. Values are mean ± SD ($n = 3$).

Inhibitory effect of amentoflavone on the microvessel formation. Incubation of aortic rings with amentoflavone showed a significant and dose dependent inhibition of microvessel outgrowth from the rat aorta ring induced by the conditioned medium from B16-F10 melanoma cells (Fig. 3), with 91.7% inhibition in microvessel out growth at a concentration of 0.2 μg/ml.

Effect of amentoflavone on endothelial cell proliferation. Amentoflavone treatment showed a significant inhibition of VEGF induced endothelial cell proliferation. Amentoflavone at a concentration of 0.2 μg/ml showed 27.7% inhibition in the proliferation of endothelial cells (Fig. 4).

Inhibition of endothelial cell migration by amentoflavone. Amentoflavone treatment showed a significant and concentration-dependent inhibition of endothelial cell migration. Amentoflavone at a non-toxic concentration of 0.2 μg/ml, showed 93.7% inhibition of endothelial cell migration. At 0.05 and 0.1 μg/ml concentrations, the percentage inhibition was found to be 12.5 and 65%, respectively (Fig. 5).

Inhibition of capillary-like structure formation by amentoflavone. Amentoflavone treatment could significantly inhibit the VEGF-induced endothelial cell tube formation in a concentration dependent manner and maximum inhibition was observed at 0.2 μg/ml (Fig. 6). Amentoflavone at concentrations 0.05 and 0.1 μg/ml also produced significant inhibition in tube formation (data not shown).

Inhibition of VEGF mRNA by amentoflavone. Amentoflavone at concentrations 0.1 and 0.2 μg/ml showed a significant inhibition of VEGF mRNA production in B16-F10 melanoma cells. At a concentration of 0.05 μg/ml, no significant inhibition in VEGF mRNA levels was observed (Fig. 7).

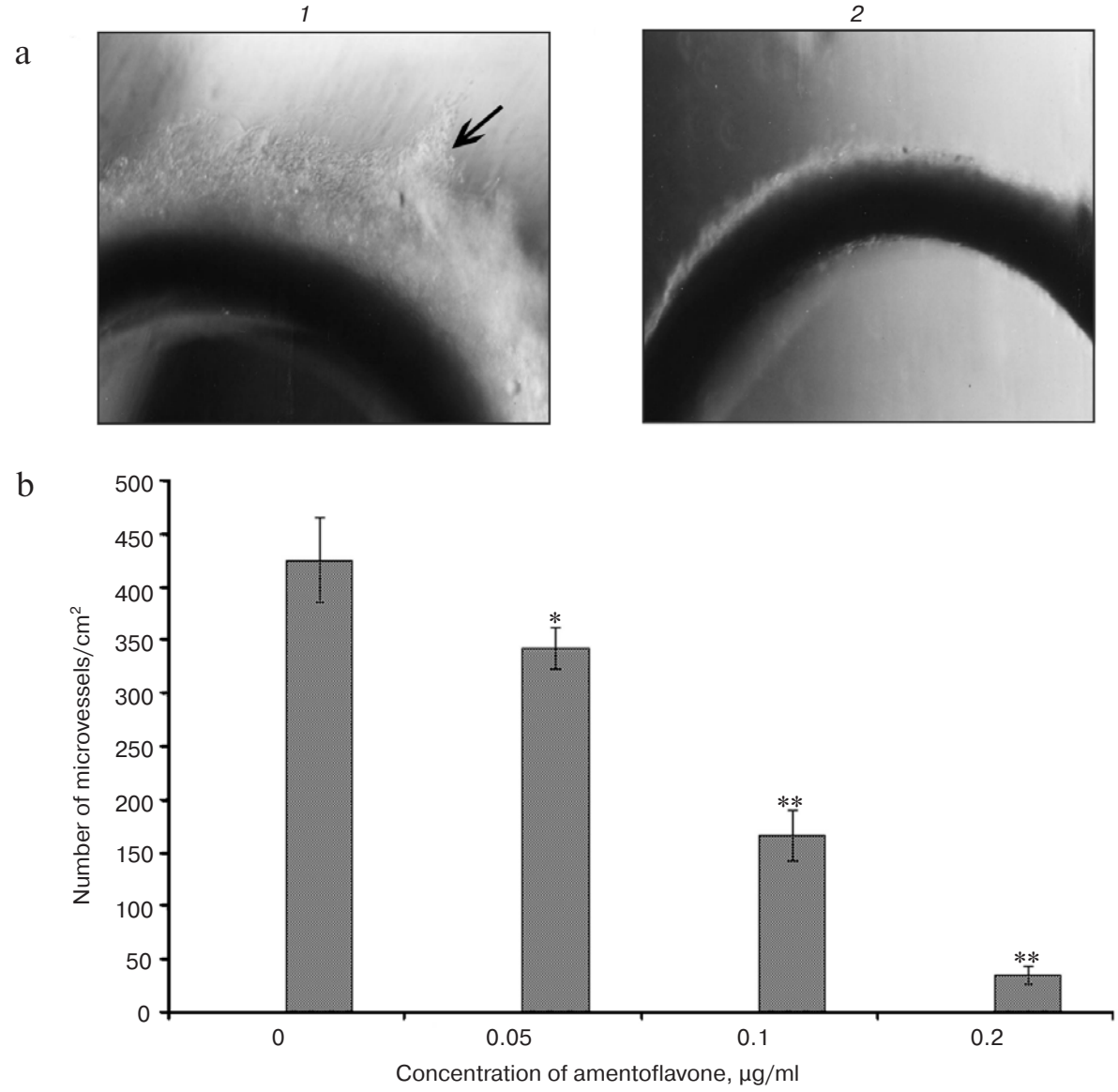

Fig. 3. Inhibition of microvessel sprouting from rat aortic ring by amentoflavone. a) Microvessel sprouting after six days in collagen matrices incubated in a conditioned medium from B16-F10 cells (*1*) and in a conditioned medium with amentoflavone at 0.2 μg/ml (*2*). b) Number of microvessels sprouting from rat aorta. Values are mean ± SD of three independent experiments. The asterisks indicate statistical significance (* $p < 0.05$, ** $p < 0.01$) as a comparison between the amentoflavone treated and the representative untreated groups. Note that microvessels were formed with the function of conditioned medium from B16-F10. The culture treated with amentoflavone (0.2 μg/ml) produced significantly fewer microvessels ($p < 0.01$) in the presence of conditioned medium.

Inhibition of angiogenesis by amentoflavone *in vivo*. Control animals had an average number of 39.9 ± 1.5 capillaries around the tumor (Fig. 8a). Amentoflavone treatment showed a significant inhibition (52.9%) in the formation of tumor directed capillaries (Fig. 8c). TNP-470 (reference compound) treatment showed 91.2% inhibition in the tumor directed neovessel formation (Fig. 8b).

Effect of amentoflavone on serum nitrite levels. Significant inhibition in nitrite production in serum was observed in animals treated with amentoflavone. On day 9, the serum nitrite level in amentoflavone-treated animals was found to be 36.2 μM, whereas the control animals had a nitrite value of 73.6 μM (Fig. 9). TNP-470-treated animals also showed significant inhibition in serum nitrite production (data not shown).

Effect of amentoflavone on serum cytokine and TIMP-1 levels. Amentoflavone treatment could significantly inhibit the production of IL-1β, IL-6, TNF-α, GM-CSF, and VEGF in tumor bearing animals (Table 1). On day 9, amentoflavone treatment significantly increased the serum IL-2 and TIMP-1 levels in tumor bearing animals to 27.6

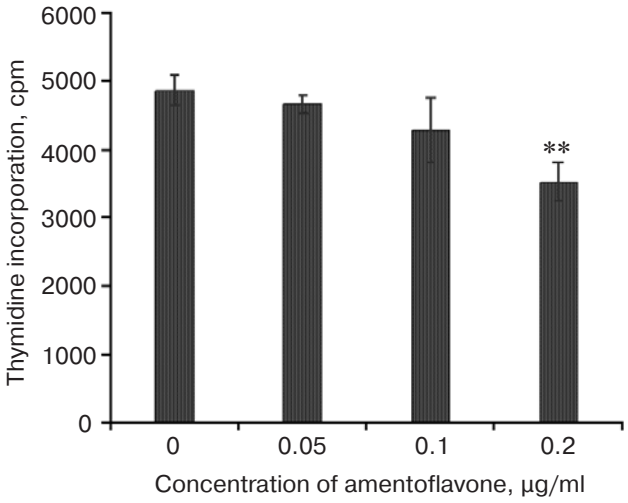

Fig. 4. Inhibition of human umbilical vein endothelial cell (HUVEC) proliferation by amentoflavone. HUVEC ($5 \cdot 10^4$ cells) were cultured for 24 h in the presence of increasing concentrations of amentoflavone (0.05-0.2 μg/ml) along with VEGF (2 ng/ml) followed by 18 h incubation after adding [^{3}H]thymidine (1 μCi). Measured radioactivity was expressed as mean (cpm) ± SD. ** $p < 0.01$, amentoflavone treated versus untreated groups. Representative of three separate experiments.

and 586 pg/ml, respectively (Table 2). TNP-470 treated animals also showed significant inhibition in serum cytokine and TIMP-1 production (data not shown).

DISCUSSION

It is widely accepted that small tumors remain dormant in the absence of angiogenesis and that tumor growth can be suppressed by inhibiting angiogenesis [30]. Neovascularization increases the probability of metastasis in part by facilitating the shedding of tumor cells into the circulation. Since angiogenesis is essential for tumor growth, inhibition of angiogenesis has a good chance in preventing cancer from becoming malignant [31, 32]. The process of angiogenesis is a critical process, which involves a concerted sequence of events including activation of endothelial cells, degradation of the proximal basement membrane, and canalization of endothelial cords penetrating surrounding tissue [33, 34]. In the present study, we for the first time provide direct evidences that amentoflavone has a potent antiangiogenic activity in the *in vitro* as well as *in vivo* models that can support its

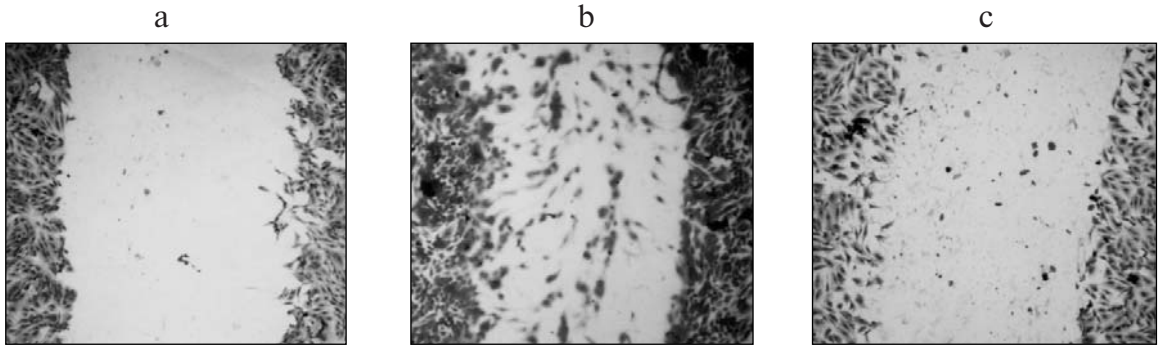

Fig. 5. Crystal violet staining of migrated endothelial cells. Wound migration assay was performed on the confluent monolayer of endothelial cells. Increasing concentrations of amentoflavone (0.05-0.2 μg/ml) along with VEGF (2 ng/ml) were added to HUVEC monolayer after creating the wound and incubated for 48 h followed by crystal violet (0.1%) staining. a) 0 h control; b) 48 h control; c) 48 h amentoflavone (0.2 μg/ml).

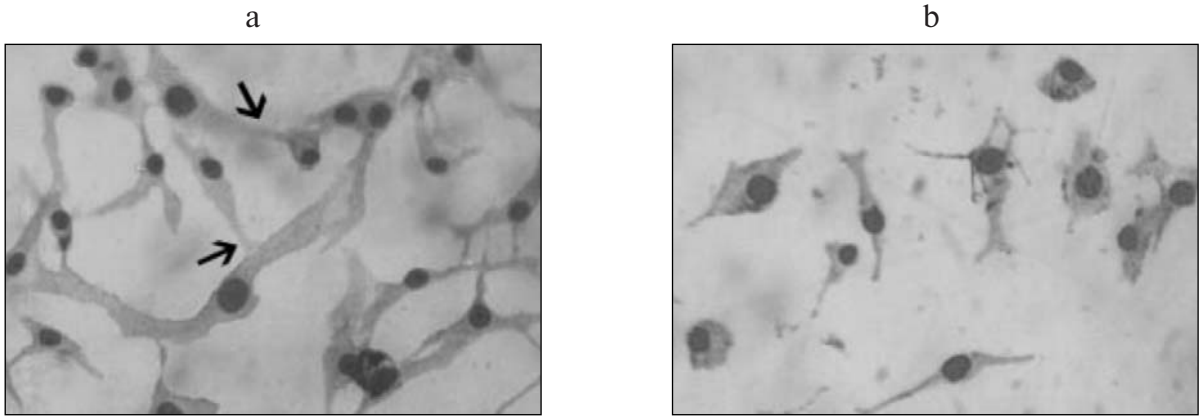

Fig. 6. Diff-Quick staining of endothelial cell tube formation. Endothelial cells ($5 \cdot 10^4$) were cultured with increasing concentrations of amentoflavone (0.05-0.2 μg/ml) and VEGF (2 ng/ml) for 48 h followed by Diff-Quick staining. a) 48 h control; b) 48 h amentoflavone (0.2 μg/ml).

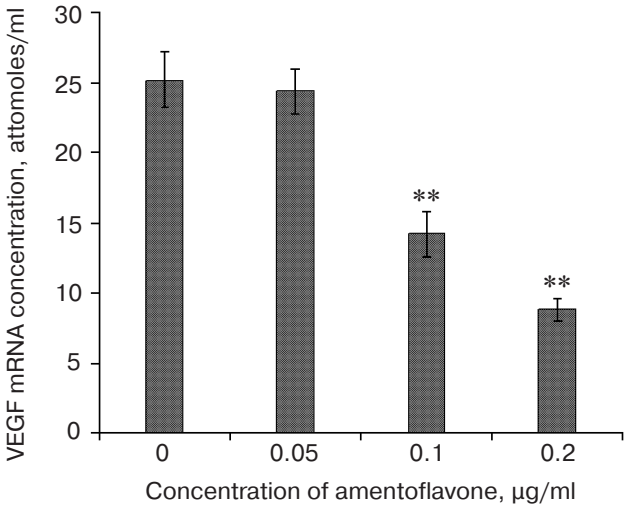

Fig. 7. Levels of VEGF mRNA expressed by B16-F10 cells. B16-F10 cells (10^6) were cultured in the presence of increasing concentrations of amentoflavone (0.05-0.2 μg/ml) and mRNA preparations were made and quantified spectrophotometrically at 490 nm. Values are expressed as mean (attomol/ml) ± SD ($n = 3$). The asterisks indicate statistic significance ($p < 0.01$) as a comparison between the treated and respective untreated groups.

tumor preventive action. In order to quantify various sequential events that occur during angiogenesis, capillary [35], aortic [36], and human umbilical vein endothelial cells [37, 38] were used in our system. Our studies in an animal model revealed that amentoflavone treatment significantly reduced the number of capillaries around the tumor. Not all the vessels in the ventral side were affected by treatment with amentoflavone, particularly larger vessels. This indicated that only newly formed capillaries are inhibited and mature vessels formed before treatment is initiated are not sensitive to amentoflavone inhibition. Decrease in microvessel was further verified by rat aortic assay. The rat aortic assay bridges the gap between the *in vivo* and *in vitro* models combining advantages from both systems. Antagonistic effects of soluble factors or matrix factors on angiogenesis can be evaluated easily using this model. Conditioned medium from B16-F10 melanoma cells promoted the growth of microvessels. This growth of microvessel was inhibited on treatment with amentoflavone, indicating its role as a promising angiogenic inhibitor. The process of angiogenesis is associated with various processes such as endothelial cell proliferation, migration, and tube formation [39]. Capillary tube formation in the basement membrane like Matrigel requires endothelial cell–matrix interactions, intercellular communications, as well as cell motility. We demonstrated that amentoflavone could inhibit the VEGF-induced migration and tube formation of endothelial cells *in vitro*. The inhibition, as observed in our study after amentoflavone treatment, might be due to reduced proliferation of endothelial cells. The concentrations of amentoflavone used in the present studies did not interfere with the viability of endothelial cells.

It has been proved conclusively that angiogenesis is essential for a tumor to become large and malignant, hence inhibition of the process of angiogenesis either by suppression of angiogenic growth factors or inhibiting endothelial cells should provide a novel therapeutic window for cancer therapy. VEGF has been identified as a key modulator of angiogenesis and most tumor cells secrete VEGF [40], and VEGF mRNA is highly upregulated in most human cancers [41]. In addition to producing VEGF themselves, tumors may induce the production of VEGF in other surrounding tissue; therefore, high levels of VEGF production in a wide variety of tumors and tumor-associated cells suggest that VEGF play a pivotal role in tumor angiogenesis. Quantification of growth factor VEGF by ELISA in our studies indicated a decreased amount of VEGF in serum of amentoflavone-treated animals compared to control animals. Our results show the presence of tumor directed capillaries in mice bearing

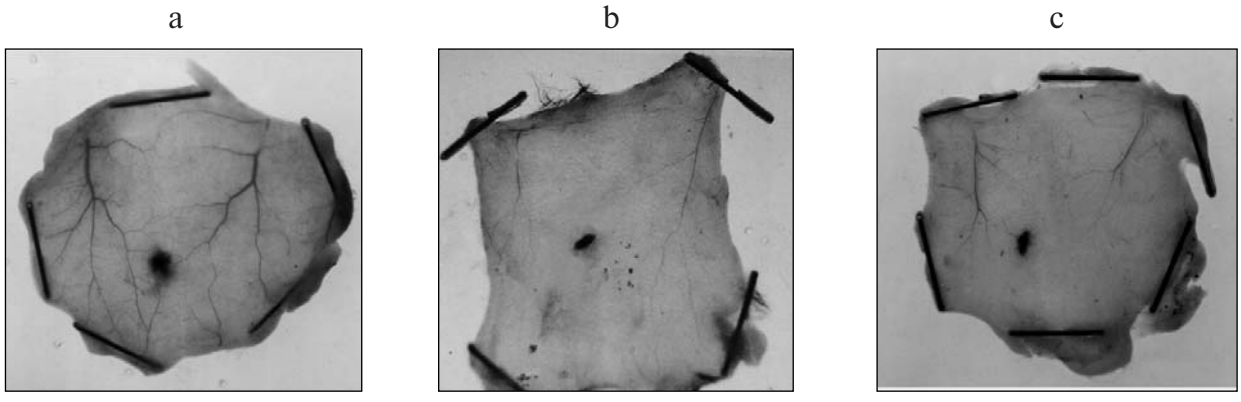

Fig. 8. Effect of amentoflavone on *in vivo* angiogenesis. Tumor angiogenesis was induced by s.c. injection of B16-F10 cells (10^6 cells/mice) on shaved ventral side of C57BL/6 mice treated simultaneously with 30 mg TNP-470 per kg body weight or 10 mg amentoflavone per kg body weight (i.p.) for five consecutive days. a) Control; b) TNP-470 (30 mg per kg body weight); c) amentoflavone (10 mg per kg body weight).

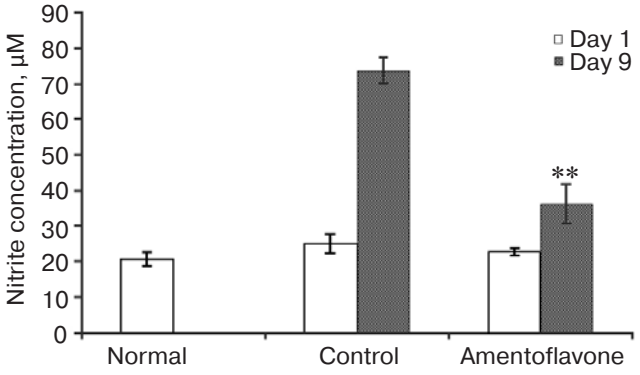

Fig. 9. Inhibition of nitrite levels by amentoflavone *in vivo*. C57BL/6 mice (six animals per group) were treated with 50 mg amentoflavone per kg body weight (i.p.) for five consecutive days after injecting B16-F10 cells (10^6, s.c.) on shaved ventral side of mice. The serum nitrite levels were determined using Griess reagent. Values are expressed as mean (μM) ± SD ($n = 6$). The asterisks indicate statistical significance ($p < 0.01$) as a comparison between the treated and control groups.

tumor cells, which might be due to the presence of VEGF in the circulation. Since there is inhibition of angiogenesis by amentoflavone, it supports the view that amentoflavone may repress the expression of VEGF-like factors or inhibit the secretion of such factors into the circulation, thereby inhibiting the formation of new blood vessels.

The process of neovascularization is a significant prognostic factor in melanoma and is controlled by several endogenous factors [42]. Different factors produced by tumor cells and surrounding stromal cells play important roles in regulating tumor angiogenesis by activating or blocking different pathways [43]. These factors include NO and several proangiogenic as well as proinflammatory cytokines such as IL-1β, IL-6, TNF-α, and GM-CSF. Therefore, it is necessary to determine the effect of amentoflavone on the production of NO and proinflammatory cytokines in tumor bearing animals. Nitric oxide synthesized by iNOS (inducible nitric oxide synthase) is a mediator involved in different processes varying from neurotransmission to affecting the vasculature. When produced by tumor cells, NO may induce vasodilation resulting in enhanced permeability in tumor vasculature. In such case, the blood flow in the tumor will be increased, which would provide a favorable condition for the tumor [44, 45]. In the present study, amentoflavone was found to inhibit nitrite levels in angiogenesis-induced animals.

Cytokines are a large family of soluble proteins participating in the communication and regulation of

Table 1. Effect of amentoflavone on the content of cytokines and VEGF (pg/ml) in animals with induced angiogenesis

Cytokines	Normal		Angiogenesis (control)		Angiogenesis + amentoflavone	
	day 1	day 9	day 1	day 9	day 1	day 9
IL-1β	16 ± 3.5	–	31.7 ± 1.0	32.5 ± 0.9	17.5 ± 3.6**	28.8 ± 2.6**
TNF-α	20 ± 3.2	–	78.0 ± 7.3	609.5 ± 8.4	68.8 ± 4.5*	174.2 ± 5.6**
IL-6	35 ± 6.5	–	41.2 ± 3.2	326.2 ± 5.5	39.4 ± 4.2	58.4 ± 6.8**
GM-CSF	18 ± 3.1	–	28.6 ± 1.9	65.4 ± 1.0	20.2 ± 2.8**	30.4 ± 4.2**
VEGF	16 ± 8	–	61 ± 5.6	156.2 ± 8.2	33.2 ± 5.2**	62.8 ± 2.3**

Note: Angiogenesis was induced by s.c. injection of B16F-10 cells (10^6) on shaved ventral side of C57BL/6 mice treated simultaneously with 10 mg amentoflavone per kg body weight (i.p.) for five consecutive days. Blood samples were collected at designated time periods from the animals with induced angiogenesis, and cytokine and VEGF levels in serum were estimated by ELISA. Data presented are mean ± SD ($n = 6$). * $p < 0.05$; ** $p < 0.01$.

Table 2. Effect of amentoflavone on the content of IL-2 and TIMP-1 (pg/ml) in animals with induced angiogenesis

Treatment	IL-2		TIMP-1	
	day 1	day 9	day 1	day 9
Normal	23 ± 3.2	–	1600 ± 36	–
Control	16.6 ± 0.7	18.2 ± 0.6	346 ± 12	362.8 ± 10.6
Amentoflavone*	22.5 ± 2.8	27.6 ± 4.2	520 ± 33	586 ± 24.5

Note: Angiogenesis was induced by s.c. injection of B16F-10 cells (10^6) on shaved ventral side of C57BL/6 mice treated simultaneously with 10 mg amentoflavone per kg body weight (i.p.) for five consecutive days. Blood samples were collected at designated time periods from the animals with induced angiogenesis, and IL-2 and TIMP-1 levels in serum were estimated by ELISA. Data presented are mean ± SD ($n = 6$). * $p < 0.01$.

inflammatory responses [46]. The cytokine system is a complicated network with cross talk between different pathways. More recently, some cytokines have been implicated as putative mediators of angiogenesis [47]. Altered levels of proinflammatory cytokines have been observed in various forms of cancer [48] including melanoma [49, 50]. The proinflammatory cytokines such as IL-1β, IL-6, TNF-α, and GM-CSF act as autocrine growth factors for tumor cells. These cytokines could be prometastatic or proangiogenic, and their deregulated expression directly correlates with the metastatic potential of several human carcinomas [51].

IL-1β has been suggested to be the primary driver of cell growth, proliferation, matrix degradation, and angiogenesis in invasive melanoma [52, 53]. Active secretion of IL-1β by tumor cells promotes their invasiveness, but the degree of invasiveness may depend upon the concentration of secreted IL-1β. *IL-1β* gene knockout mice showed lesser degree of microvessel formation compared to its wild type when induced with B16 melanoma [54]. Increased invasiveness of IL-1β-secreting cells was shown to be mediated by enhanced tumor angiogenesis and increased angiogenic factor production (vascular endothelial growth factor, macrophage-inflammatory protein 2) through communication networks between the malignant cells and stromal cells [55]. In our experiment, amentoflavone treatment was found to inhibit the IL-1β production in angiogenesis-induced animals. Thus amentoflavone mediated inhibition of IL-1β may contribute to diminished tumor angiogenesis, probably because secreted IL-1β activates, by autocrine/paracrine manners, an inflammatory cascade in the tumor's microenvironment, and this needs further investigation.

Proinflammatory mediators, such as TNF-α, are known to activate the endothelium and increase the expression of adhesion molecules and chemokines, which are crucial steps of leukocyte recruitment [56]. The recruited leukocytes can mediate angiogenesis by release of endothelial growth factors [57, 58]. TNF-α is among the most potent inducers of transcription factor NF-κB, which has been recognized as a major regulator of pathogen and inflammatory-inducible gene regulation. TNF-α exerts its regulatory effect on both iNOS and VEGF and has been found to upregulate MMP-9 expression and thereby angiogenesis [59, 60]. Treatment with amentoflavone could inhibit the TNF-α level in angiogenesis-induced animals, thus preventing the tumor-directed new blood vessel formation by downregulating other angiogenic molecules.

Interleukin-6 (IL-6) is an important cytokine, which plays an important role in thrombocytosis and also in the storage of VEGF in thrombocytes [61]. Vascular endothelial cell growth factor (VEGF) is an endothelial cell-specific mitogen proved to play a key role in neovascularization [62, 63]. IL-6 has been shown to upregulate VEGF-A load in the platelet aggregate on tumor endothelium [64]. Serum IL-6 and VEGF levels were positively correlated in advanced cancers [61]. In our study, amentoflavone treatment was shown to inhibit the production of IL-6 accompanied with reduced levels of VEGF in the circulation. Since IL-6 has been shown to activate several signaling pathways, including the Janus-activated kinase/signal transducer and activator of transcription 3 (Jak-STAT 3), phosphoinositol-3-kinase (PI-3K), and mitogen activated protein kinase pathways [65-67], amentoflavone treatment might have interfered with the IL-6-mediated signaling, thereby affecting the integrity of thrombocytes and angiogenesis.

Granulocyte monocyte colony stimulating factor (GM-CSF) is a pleiotropic cytokine produced by a number of different cell types such as macrophages, T-cells, granulocytes, fibroblasts, endothelial cells, and various carcinoma cells. Even though GM-CSF did not modulate endothelial cell functions related to inflammation [68], it induces endothelial cells to proliferate and migrate. Tumors expressing GM-CSF have been shown to have megakaryocyte potentiating activity due to IL-6 and cause thrombocytosis [69]. Since thrombocytes release VEGF into the circulation, thereby promoting angiogenesis, inhibition of GM-CSF, by amentoflavone treatment might open novel molecular properties that interfere with common angiogenic signaling pathways triggered on VEGF release by thrombocytes.

Interleukin-2 was the first cytokine used clinically for treating cancer [70], and there are reports that IL-2 promotes the proliferation and differentiation of helper T-cells, cytotoxic-T-cells and B-cells, which augments innate or natural immunity by stimulating NK cells [71]. NK cells activated with IL-2 can adhere to and lyse endothelial cells [72]. Increased production of IL-2 in angiogenesis-induced animals was observed after amentoflavone treatment. The increased production of IL-2 could successfully shift the equilibrium towards angiostatic condition.

Natural inhibitor proteins, such as tissue inhibitor of metalloproteases (TIMPs), may function as metastasis suppressor proteins by inhibiting matrix metalloproteases (MMPs). TIMP-1 is a specific inhibitor of MMP-9. An imbalance between protease and inhibitor levels has been reported to accompany tumor angiogenesis [73]. During the activation phase of angiogenesis, increased expression of TIMPs inhibits tumor-associated angiogenesis. However, inhibition of MMP activity is required in the resolution phase of angiogenesis to stabilize newly formed blood vessels and allow basement membrane deposition [74]. In our experiments, inhibition of angiogenesis on amentoflavone treatment is accompanied with high levels of TIMP-1, suggesting that amentoflavone might have altered the balance between MMPs and their inhibitors in favor of proteolytic activity.

All the above results indicate that amentoflavone possess very good antiangiogenic qualities and could

inhibit the production of cytokines such as IL-1β, TNF-α, IL-6, GM-CSF, and the growth factor VEGF. This inhibition may be attributable to the interference with signaling cascade, which may inhibit the expression of several proinflammatory cytokines. Recently we had shown that amentoflavone could inhibit the activation and nuclear translocation of transcription factors such as NF-κB p65, NF-κB p50, NF-κB c-Rel, c-Fos, ATF-2, and CREB in B16-F10 melanoma cells [75]. Further studies have to be conducted to elucidate the mechanism of action of amentoflavone in regulation of transcription factors during the angiogenesis process.

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