CHAPTER 6

Effects of bleomycin on *in vitro* angiogenesis

6.1. Introduction

Intralesional bleomycin has been cited as a safe and effective therapeutic modality for haemangiomas. [1,2,3] Although the favourable results observed following the treatment of these tumours with bleomycin were attributed to the sclerosing effect of the drug on vascular endothelium, experiments undertaken earlier in this study showed that bleomycin inhibited neovessel formation in cultured haemangioma biopsies. Since haemangiomas are characterized by aggressive angiogenesis in the proliferative phase, [4,5,6] it seems plausible that bleomycin could inhibit haemangioma growth by inhibiting angiogenesis.

According to Montesano *et al*. (1986), angiogenesis is marked by several important events which include the production of enzymes capable of degrading the extracellular matrix, as well as endothelial cell migration and proliferation. [7] Following their migration and proliferation in the interstitial stroma, endothelial cells align and establish contact with each other, originating capillary-like structures. [8] This event, referred to as capillary morphogenesis, has been recapitulated *in vitro* using assays in which endothelial cells seeded on a permissive matrix substrate organize to form tubular structures resembling capillary blood vessels. [9,10] In some models, vascular endothelial growth factor and fibroblast growth factor have been used to induce cultured endothelial cells to form capillary-like structures. [7] These growth factors have also been implicated in the pathogenesis of haemangiomas. [13]

In this study the effects of bleomycin on VEGF and bFGF-induced *in vitro* angiogenesis were investigated using a three-dimensional collagen gel model. The effects of drugs previously reported to inhibit aspects of the angiogenesis process (mitomycin C, taxol, 2-methoxyestradiol, vincristine, vinblastine, colchicine, nocodazole and cytochalasin D) [14-18], were also investigated using the same model.

6.2. Materials and Methods

6.2.1. Endothelial Cell Culture

Adrenal cortex-derived bovine microvascular endothelial (BME) cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The cells were grown in α-modified minimum essential medium (α-MEM) supplemented with 15% heat-inactivated donor calf serum (DCS), penicillin (500 U/ml) and streptomycin (100 µg/ml).[16] The *in vitro* angiogenesis assay was performed using BME cells as previously described, [17, 18] and the work was undertaken in Prof Michael Pepper's laboratory, University of Geneva, Switzerland.

6.2.2. Preparation of Collagen Gels

Type 1 collagen was solubilised by stirring adult rat tail tendons for 48h at 4°C in sterile 1:1000 (vol/vol) acetic acid solution (300 ml for 1mg of collagen). The resulting solution was filtered through a sterile triple gauze and centrifuged at 16 000x g for 1 hour at 4°C. Eight volumes of the supernatant were mixed with 1 volume of 10x minimum essential medium and 1 volume of sodium bicarbonate (11.76 mg/ml) in a sterile flask placed on ice, to prevent immediate gelation. The cold mixture was then dispensed into 18 mm tissue culture wells and allowed to gel for 10 min at 37°C.

6.2.3. *In Vitro* Angiogenesis Assay

Bovine microvascular endothelial cells were seeded onto collagen gels at 1.0 x 10^5 cells/well in 500 µl α-MEM supplemented with 5% DCS, and were grown to confluence (3-4 days), at which point treatment with growth factors and/or drugs was begun. Medium and treatments were renewed on the second and fourth day. After six days, cultures were fixed with 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.4). Fixed cultures were photographed for quantification of invasion.

6.2.4. Quantification of Invasion

Three randomly selected fields measuring 1.0 x 1.4 mm were photographed in each well at a single level beneath the surface monolayer by phase contrast microscopy. Invasion was quantitated by determining the total additive sprout length in µm ± SEM, and results are from at least two separate experiments.

6.3. Statistical Analysis

Statistics were performed using Stata Release 8. One-way ANOVA was employed, and where statistical significance was reached, pair-wise multiple comparisons were made using Bonferroni's method. When data were not normally distributed ANOVA on ranks was performed, followed by pair-wise comparisons again employing the Bonferroni approach. Significance was designated at $P<0.05$.

6.4. Results

6.4.1. *In vitro* angiogenesis

Untreated BME cells formed a monolayer on the surface of the gel (fig 6.1 A); BME cells treated with a combination of 10 ng/ml bFGF and 30 ng/ml VEGF (fig 6.1 B) organized into branching cords inside the collagen matrix. Fewer such cords were observed in cultures treated with a combination of growth factors and 30µg/ml bleomycin (fig 6.1 D). No cords were observed in cultures treated with 100 µg/ml bleomycin (fig 6.1 F), however, cells formed a confluent monolayer, and appeared similar to control cells.

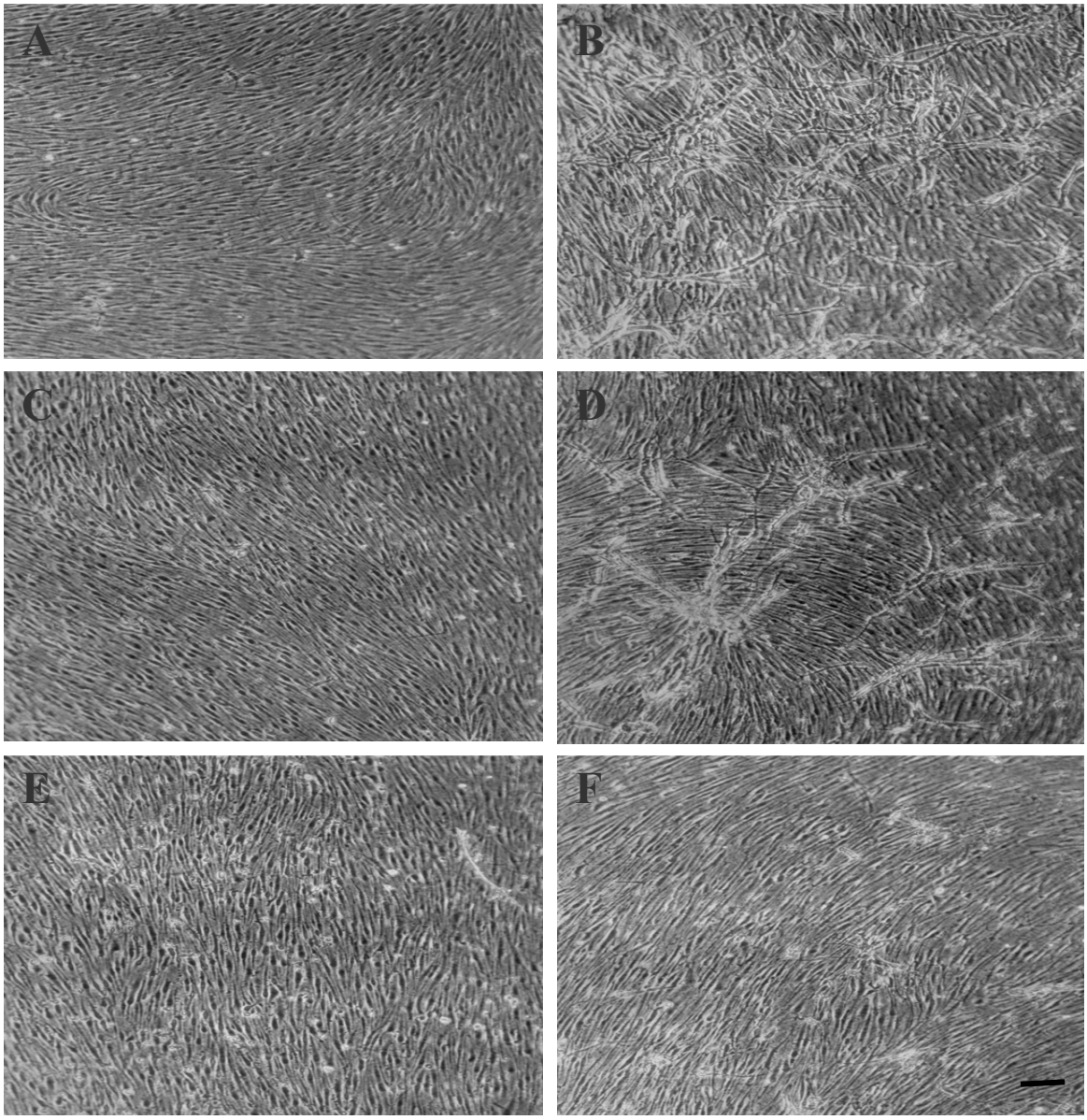


Figure 6.1. Phase contrast views of the effects of bleomycin on bovine microvascular endothelial (BME) cells grown on collagen gel. (A) – untreated cells; (B)- cells treated with a combination of 30 ng/ml VEGF + 10 ng/ml bFGF. Cells in (C) and (E) were treated with 30 µg/ml + 100 µg/ml bleomycin respectively; while cells in D and F were treated with 30 µg/ml (D) and 100 µg/ml (F) of bleomycin in the presence of growth factors. Bar = 100 µm

Control BME cells formed a confluent monolayer on the surface of the gel (figure 6.2 A); BME cells exposed to 10ng/ml bFGF invaded the underlying gel matrix and formed capillary-like tubular structures (B); there were no such structures in cultures exposed to 2-ME, taxol (fig 6.2 C and E) or colchicine (fig 6.3 C); cells appeared polygonal in morphology. No endothelial cell cords were observed in cultures treated with a combination of bFGF and 2-ME (fig 6.2 D) or taxol (fig 6.2 E). Few scattered endothelial cell cords were observed in bFGF-treated cultures exposed to colchicine (fig 6.3 F).

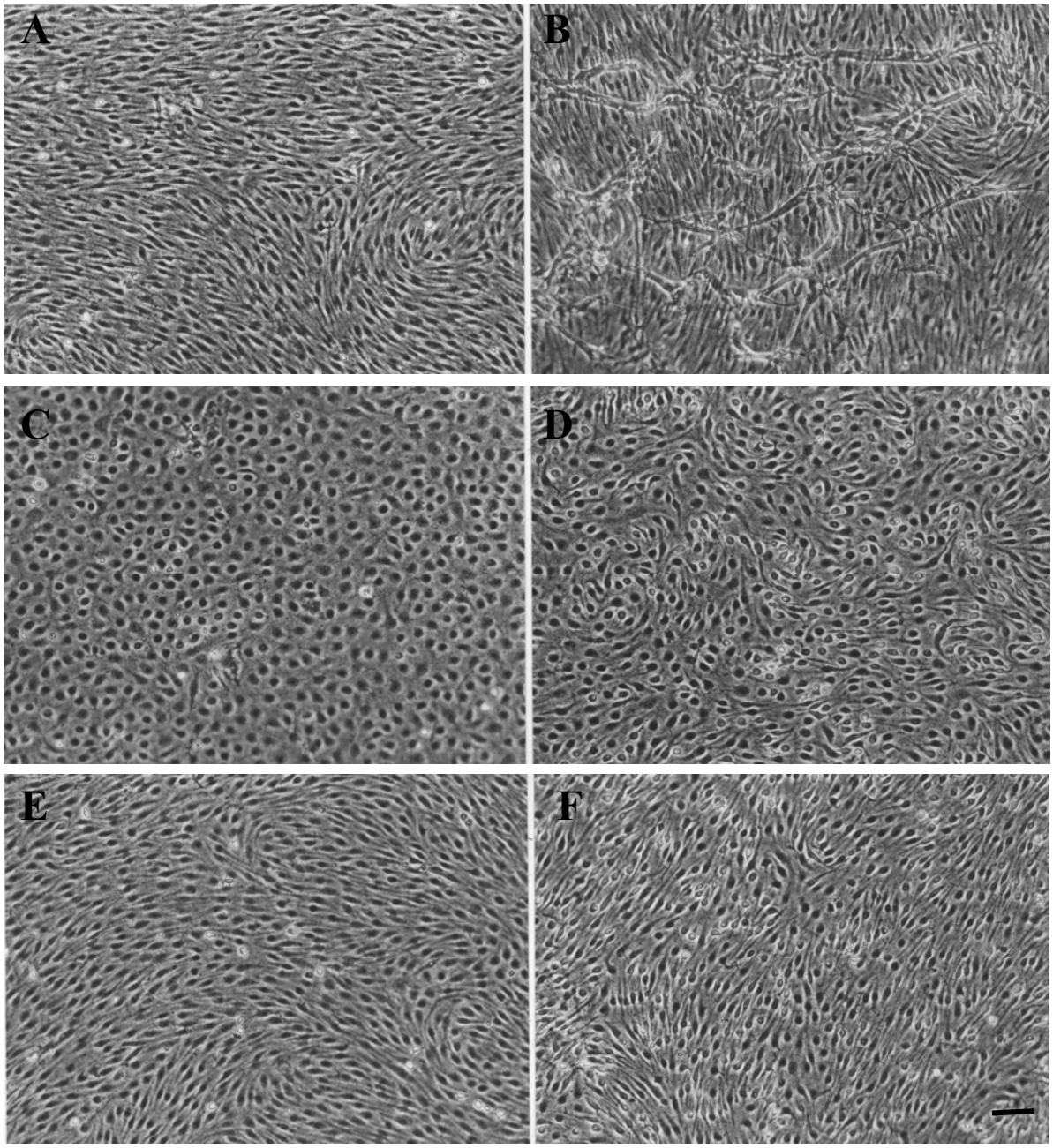


Figure 6.2. The effects of 2-methoxyestradiol (2-ME) and taxol on BME cells grown on collagen gel (Phase contrast). (A) Control untreated cultures; (B) bFGF-treated cultures; (C) 2-ME- and (E) Taxol-treated cultures; Cultures treated with a combination of bFGF and either (D) 2-ME or (F) Taxol. Bar = 100 µm

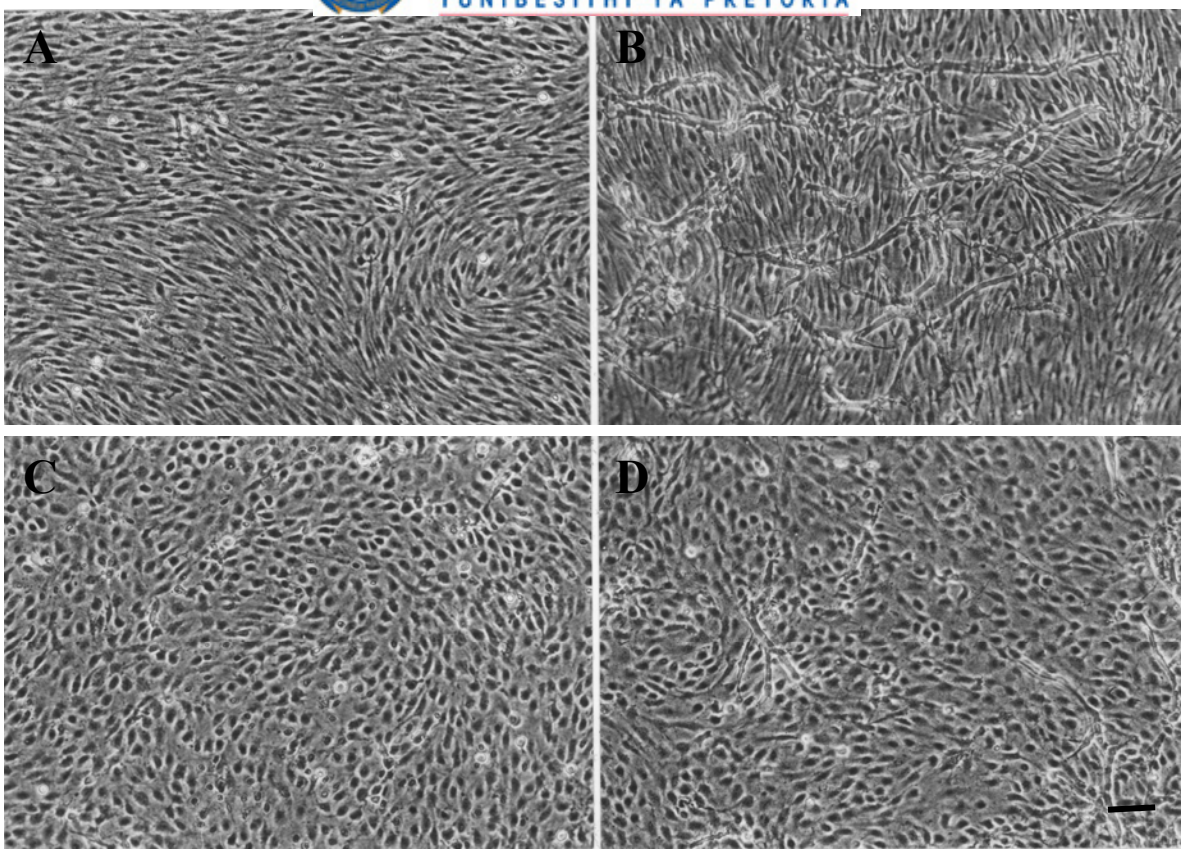
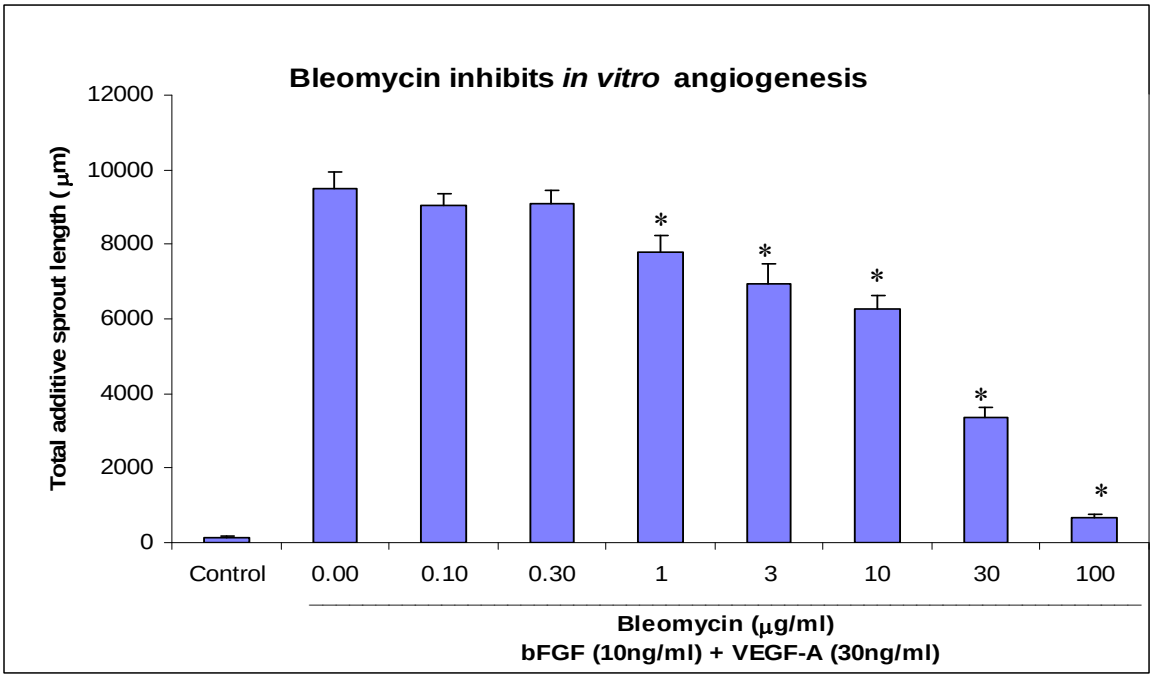


Figure 6.3. Effect of colchicine on BME cells grown on collagen gel (A and B are the same as in fig 6.2). (A) Control BME cells; (B) BME cells exposed to 10ng/ml bFGF; (C) Cultures exposed to colchicine only; (D) bFGF-treated cultures exposed to colchicine. Bar = 100 µm

6.4.2. Quantification of invasion

Cultures either received no treatment, were treated with a combination of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), or were treated with a combination of growth factors and varying doses of bleomycin or mitomycin C. Quantitative analysis revealed that both bleomycin and mitomycin C inhibited growth-factor induced *in vitro* angiogenesis in a dose-dependent manner, with IC_{50} values of 17.78 and 0.79 µg/ml respectively (table 6.1).



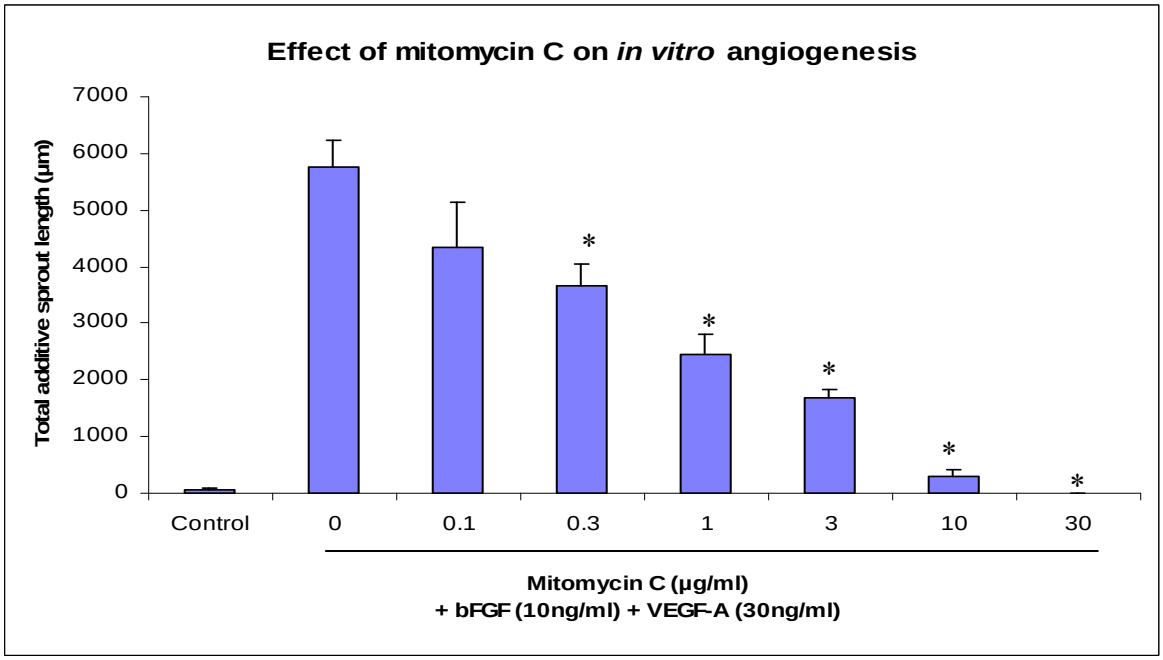
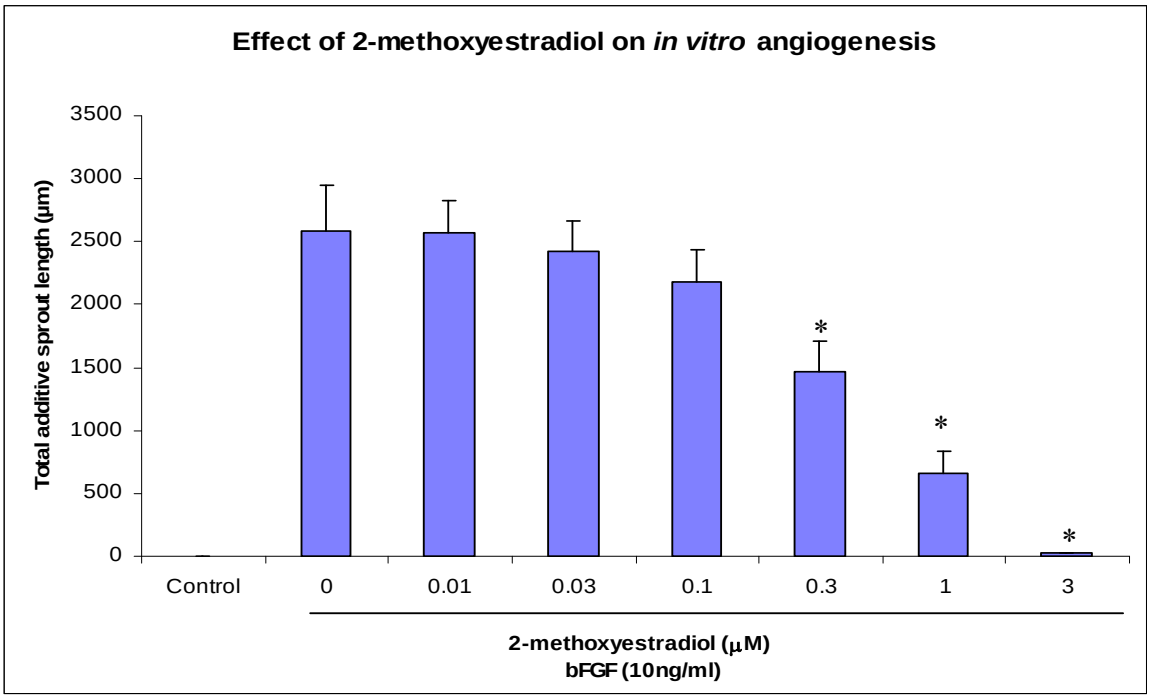


Figure 6.4. Quantitative analysis of the effects of bleomycin and mitomycin C on *in vitro* angiogenesis. Significant difference in the total additive sprout length between bFGF only -treated (0 µg/ml drug) and cells treated with a combination of bFGF + drug: * $P < 0.05$

Basic fibroblast growth factor (bFGF) was employed to induce endothelial cell cord formation. Both growth factors have been previously shown to induce angiogenesis *in vitro* singly or in combination, and therefore for further studies bFGF alone was employed. 2-ME and taxol inhibited bFGF-induced endothelial cell cord formation with IC_{50} values of 0.4 and 0.01 µM respectively (table 6.1).



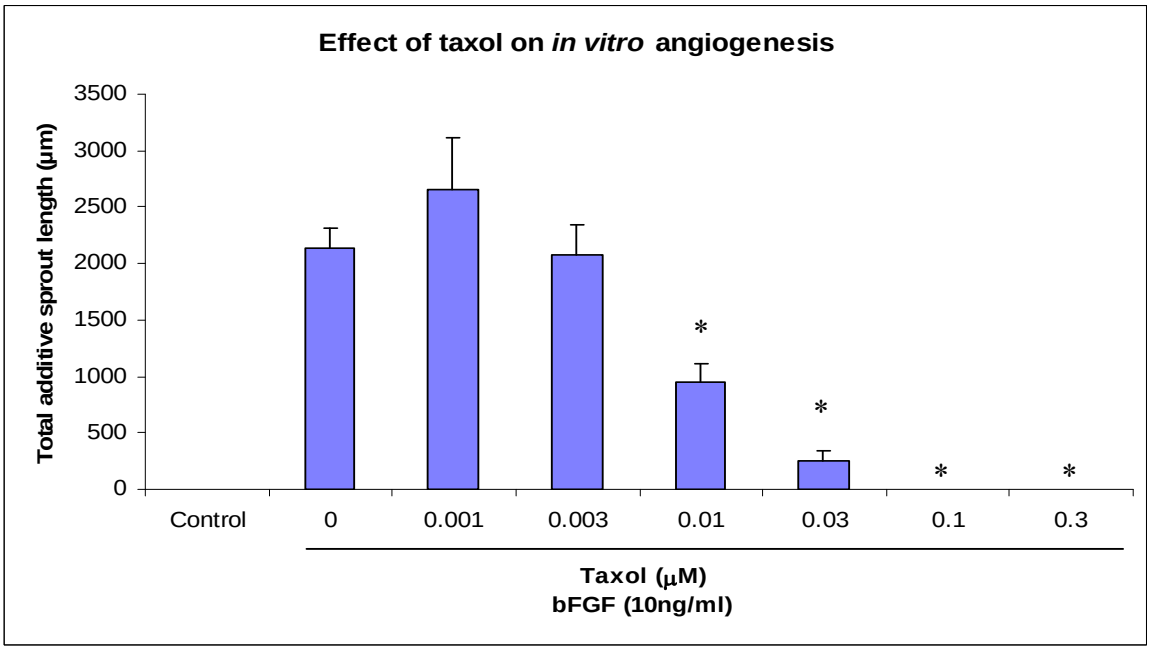
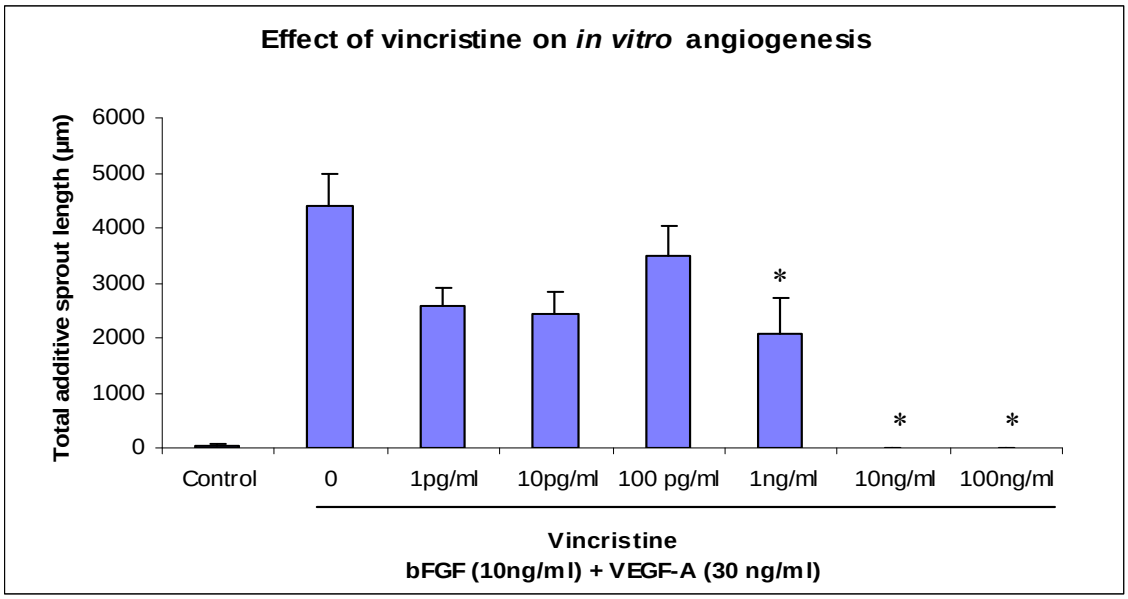


Figure 6.5. Quantitative analysis of the effects of 2-methoxyestradiol and taxol on *in vitro* angiogenesis. Significant difference in total additive sprout length between bFGF-treated (0 µM drug) and cells treated with a combination of bFGF and drug: * $P < 0.05$

Significant inhibition of bFGF-induced endothelial cell tube formation was observed at doses of 1 ng/ml for both vincristine and vinblastine.



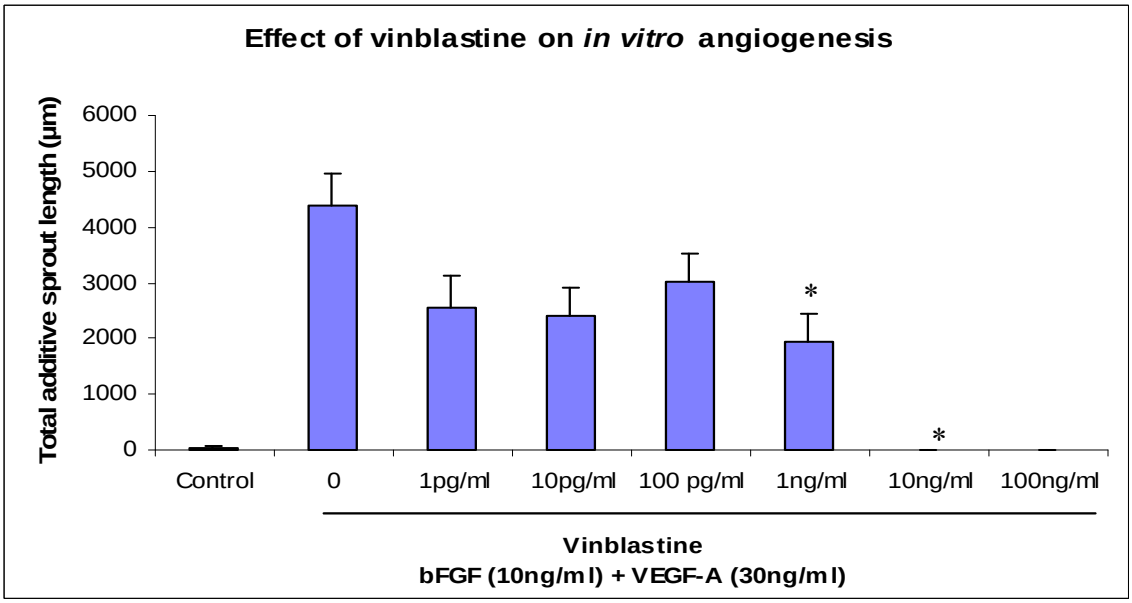


Figure 6.6. Quantitative analysis of the effects of vincristine and vinblastine on *in vitro* angiogenesis. Significant difference in total additive sprout length between bFGF only - treated (0 pg/ml drug) and cells treated with a combination of bFGF and drug: * $P < 0.05$.

Colchicine, nocodazole, and cytochalasin D inhibited the formation of capillary-like tubes by BME cells in a dose-dependent manner, with IC50 values of 0.016 for colchicine and 0.017 for both nocodazole and cytochalasin D (table 6.1).

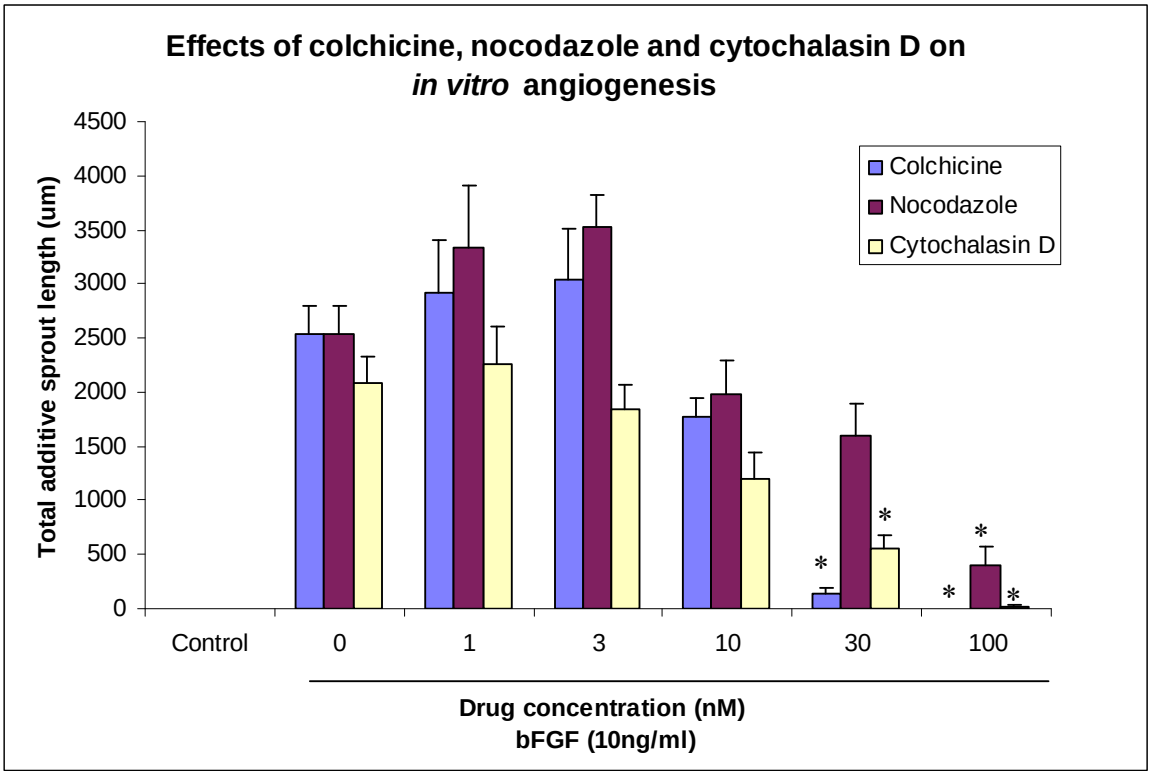


Figure 6.7. Quantitative analysis of the effects of colchicine, nocodazole and cytochalasin D on *in vitro* angiogenesis. Significant difference in total additive sprout length between bFGF only-treated (0 nM drug) and cells treated with a combination of bFGF and drug (colchicine, nocodazole or cytochalasin). * $P < 0.05$.

Table 6.1. The effects of test drugs on *in vitro* angiogenesis. IC_{50} values refer to the drug concentration at which 50% inhibition of capillary-like tube formation was observed.

Drug	**Cell Type**	**IC_{50}**
Bleomycin	BME cells	11.85 µM (17.78 µg/ml)
Mitomycin C	BME cells	2.34 µM (0.79 µg/ml)
2-methoxyestradiol	BME cells	0.40 µM
Taxol	BME cells	0.01 µM
Vincristine	BME cells	0.86 nM (0.79 ng/ml)
Vinblastine	BME cells	0.87 nM (0.79 ng/ml)
Colchicine	BME cells	0.016 µM
Nocodazole	BME cells	0.017 µM
Cytochalasin D	BME cells	0.017 µM

6.5. Discussion

The effects of bleomycin, mitomycin C, taxol, 2-methoxyestradiol, colchicine, nocodazole, cytochalasin D, vincristine and vinblastine were investigated using an assay of *in vitro* angiogenesis. This is the first comparative study on the effects of these drugs on *in vitro* angiogenesis in the same system.

Bovine microvascular endothelial cells were cultured on the surface of a three–dimensional gel of type 1 collagen, a major component of the perivascular extracellular matrix. Under normal conditions, BME cells are confined to the surface of the gels, forming a confluent monolayer. Growth factors (bFGF alone or in combination with VEGF) induced BME cells to invade into the underlying collagen matrix, where they formed extensive networks of endothelial cell cords. Electron microscopy revealed that these cords were formed by endothelial cells that had organised around a lumen, forming capillary-like tubular structures within the collagen matrix. [19] In previous studies, it was shown that bFGF and VEGF induced BME cell invasion and cord formation in a collagen gel invasion assay, and that co-addition of the two cytokines had a synergistic although not additive effect in stimulating angiogenesis *in vitro*. [19,20]

Bleomycin inhibited growth factor induced BME cell invasion of the collagen matrix in a dose-dependent manner, with an IC_{50} of ~11.85 µM (table 6.1). Another antitumour antibiotic, mitomycin C, also inhibited growth factor-induced endothelial cell invasion, with an IC_{50} attained at a lower dose (~2.34 µM). Both bleomycin and mitomycin C significantly inhibited endothelial cell cord formation at doses higher than those producing significant inhibition of BME cell growth. In a previous study, bleomycin had a marginal effect on endothelial cell growth, while mitomycin C was more potent in inhibiting endothelial cell growth. Also, in another study mitomycin C was shown to inhibit DNA synthesis and cause cell cycle arrest in endothelial cells. [21] It is likely that these inhibitory effects on endothelial cell proliferation could, in part, be responsible for the drug's inhibitory effect on *in vitro* angiogenesis.

Taxol, a microtubule-stabilizing antineoplastic cytotoxic drug, significantly inhibited bFGF-induced tube formation by BME cells. Previously, taxol was shown to inhibit endothelial cell invasion *in vitro*. [15] Taxol was also reported to inhibit the angiogenic response produced by tumour cell supernatants injected (embedded in a matrigel) into mice. [15]

In a previous report, inhibition of endothelial cell invasiveness occurred at drug concentrations which did not affect endothelial cell proliferation. [22] In this study, significant inhibition of capillary morphogenesis was observed at doses far lower than those that induce inhibition of cell growth. From these observations, it appears that the antiangiogenic activity of taxol is not linked to its cytotoxic effect. The implication is that this drug may have therapeutic potential against diseases characterised by excessive angiogenesis, and could be administered *in vivo* at much lower doses than those used in anti-cancer therapy.

2-Methoxyestradiol also inhibited bFGF-induced tube formation by BME cells, although with less potency when compared to taxol. Previously, 2-ME was shown to have anti-migratory effect on bovine brain capillary endothelial cells and BME cells, while in vivo, 2-ME was shown to inhibit bFGF- and VEGF-induced neovascularization in mice. [16,23]

The *in vitro* concentrations of colchicine in the 30 nM range had a significant inhibitory effect on angiogenesis *in vitro*, and doses of 100 nm had a more pronounced effect on growth factor-induced tube formation, completely blocking the angiogenic response. In a study conducted by Stafford (2005), colchicine exhibited antiangiogenic activity in an *in vitro* fibrin-thrombin clot- based assay at doses of 0.01 to 1µM. [23]

Nocodazole and cytochalasin D had a significant inhibitory effect on *in vitro* angiogenesis ($P < 0.05$). In a previous study, disruption of microfilaments by cytochalasin D led to inhibition of endothelial cell growth, while microtubule-disruption by nocodazole induced partial inhibition of endothelial cell growth. [24] It is possible that inhibition of *in vitro* angiogenesis by both nocodazole and cytochalasin D could be due, in part, to inhibition of BME cell growth.

Vincristine and vinblastine inhibited in vitro tube formation with comparable potency, with maximum inhibition occurring at 10 ng/ml for both drugs. In a previous study using the chorioallantoic membrane assay, it was demonstrated that both drugs had an inhibitory effect on vascularisation. In another study, vinblastine reversibly inhibited endothelial cell growth at much lower doses (0.25 pM). [15,20] Vinblastine was also shown to inhibit endothelial cell chemotaxis, the secretion of matrix-metalloproteinases (MMP-2 and -9), and capillary morphogenesis in reconstituted basement membrane. [15]

Taken together, results from this study and observations from previous studies indicate that vinca alkaloids are potent inhibitors of angiogenesis. Furthermore, vincristine, which has been employed to treat a few patients with juvenile haemangiomas, may accelerate haemangioma involution by inhibiting angiogenesis.

In conclusion, the results demonstrated that test drugs inhibited growth factor-induced BME cell invasion of the underlying collagen gel matrix and formation of capillary-like tube structures, important features in the process of angiogenesis. Since excessive angiogenesis has been cited as a central mechanism underlying the development of haemangiomas, the *in vitro* antiangiogenic effects of these test drugs warrant further investigation in an animal haemangioma model.

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