The effects of bleomycin, mitomycin C, and cytoskeletal-disrupting drugs on angiogenesis *in vitro* and haemangioma development *in vivo*

by

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Peaceful Mabeta was born in Boksburg. She obtained a BSc degree (Biochemistry and Physiology) from Medunsa. She obtained a masters degree in Physiology in 2002 from the University of Pretoria, where she currently holds a lectureship position.

Her thesis is entitled **The effects of bleomycin, mitomycin C, and cytoskeletal-disrupting drugs on angiogenesis *in vitro* and haemangioma development *in vivo***. Haemangiomas are tumours of the vasculature commonly encountered in pediatrics. The treatment of these tumours has over the years remained unsatisfactory. Recently in South Africa, intralesional bleomycin therapy has been used to treat haemangiomas with promising success. However, there is very little understanding of its mechanism of action. The candidate developed a rapid and sensitive HPLC method for the measurement of bleomycin in human plasma, and demonstrated a negligible systemic spill-over of the drug following intralesional therapy in haemangioma patients. In her thesis, Ms Mabeta showed that bleomycin inhibits haemangioma growth in part by inhibiting angiogenesis. The candidate also showed that cytoskeletal-disrupting drugs with antiangiogenic activity effectively inhibit haemangioma growth in a syngeneic mouse model, thereby supporting the notion that these drugs have potential in the treatment of these tumours. Further investigation of the therapeutic potential of these drugs in the treatment of pediatric haemangiomas is underway. The examiners allocated a mark of more than 80% to the thesis.

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Summary

Angiogenesis, the process of new vessel formation, appears to be a central mechanism that underlies the development of haemangiomas. Recently, intralesional bleomycin injection was used to treat paediatric haemangiomas with very good results. The purpose of this study was to determine whether there was significant systemic circulatory spill-over of bleomycin in haemangioma patients treated with intralesional bleomycin to determine safety of use. Furthermore, in order to elucidate bleomycin's mechanism of action in inducing haemangioma regression, this study aimed at determining the effects of bleomycin on aspects of angiogenesis, namely, endothelial cell migration, growth and apoptosis, and comparing these effects with those of drugs previously reported to inhibit various aspects of the angiogenic process (mitomycin C, 2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole and cytochalasin D). Lastly, the effects of bleomycin, mitomycin C, 2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole and cytochalasin D were studied in an animal haemangioma model.

A rapid and highly sensitive high performance liquid chromatographic (HPLC) method was developed. Blood samples were collected from four haemangioma patients before and after (over a 24 hour period) intralesional bleomycin (IB) therapy. As a control, blood samples were also collected at identical time intervals from four patients undergoing intravenous (IV) bleomycin chemotherapy for various malignant tumours. The HPLC method was used to quantitate bleomycin fractions in patient samples. The mean bleomycin concentration detected in plasma samples obtained from IB treated patients was 0.00 µg/ml for both bleomycin A_2 and B_2 over the 24-hour period following therapy. Plasma bleomycin A_2 and B_2 levels of 360.79 and 158.85 µg/ml respectively were detected in samples obtained from cancer patients treated with bleomycin IV. These findings indicate that the low levels detected may translate to a significantly lesser risk of pulmonary fibrosis following IBI.

The effect of drugs on endothelial cell migration was analyzed by wounding a confluent monolayer of cells and determining the number of cells that had migrated from the

wound edge. Endothelial cell growth was determined in cells treated with various drug concentrations while apoptosis was examined using hematoxylin and eosin staining, DNA fragmentation assay and acridine orange staining.

The effect of test drugs on *in vitro* angiogenesis was determined on endothelial cells induced to form capillary-like tubes in collagen gel. Test drugs were then evaluated for antitumour activity in an animal haemangioma model.

Data demonstrated that test drugs inhibited endothelial cell migration, with the exception of mitomycin C. All test drugs induced a reduction in the percentage of viable endothelial cell in a dose-dependant manner, and also induced endothelial cell apoptosis. The drugs inhibited angiogenesis *in vitro* and inhibited tumour development *in vivo* with varying potency.

In general, results from this study indicated that there was negligible systemic spill-over of bleomycin following IB administration in patients with haemangiomas, suggesting a much lesser risk of developing bleomycin-induced pulmonary fibrosis. This study also showed that test drugs inhibited angiogenesis *in vitro* and haemangioma development *in vivo* in a mouse model. Taken together, these observations demonstrate that bleomycin may inhibit haemangioma growth by inhibiting angiogenesis. In addition, mitomycin C, 2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole and cytochalasin D may have potential in the treatment of haemangiomas of infancy, and should be investigated further in a murine haemangioma model to determine effective dose schedules.

Keywords: bleomycin; cytoskeletal-disrupting agents; angiogenesis; haemangioma; endothelial cells; cell growth; cell migration; vascular endothelial growth factor; basic fibroblast growth factor; polyoma middle T oncogene; vascular tumour.

Opsomming

Angiogenese, die proses waarby nuwe bloedvate gevorm word, blyk om die sentrale meganisme onderliggend tot die vorming van hemangiomas te wees. Onlangs is bleomisien binne-letsels ingespuit om pediatriese hemangiomas te behandel, met baie goeie resultate. Die doel van hierdie studie was om te bepaal of daar ‘n noemenswaardige oorvloei van bleomisien in die sirkulatoriese stelsel van pasiënte waar daar bleomisien binne-in die letsel gespuit is, was, en om sodoende die veiligheid daarvan te kan bepaal. Verder, om die meganisme van aksie van bleomisien op hemangioma regressie te verduidelik indien die studie ook die effekte van bleomisien op endoteelselmigrasie, groei en apoptose met betrekking tot angiogenese, met die effek van middels wat al voorheen beskryf is, vergelyk (mitomycin C, 2-methoxyestradiol, taxol, vincristine, vinblastine, cochisine, nocodazole and cytochalisin D). Ten laaste, die effekte van bogenoemde middels is ook in ‘n hemangioma diere (muis) model bestudeer.

‘n Vinnige en hoogs sensitiewe HPLC metode is ontwikkel. Bloed monsters is van pasiënte met hemangiomas geneem, beide voor en na (oor ‘n 24 uur periode) binne-letsel inspuiting van bleomisien (IB). As kontrole, is bloedmonsters van pasiënte wat intraveneuse (IV) sistemiese bleomisien chemoterapie ontvang het vir kwaadaardige tumors, geneem. Die HPLC metode is gebruik om die vlak van bleomisien in die monsters van pasiënte te bepaal. Die gemiddelde bleomisien konsentrasie van die plasma monsters van IB behandelde pasiënte was 0.00 μg/ml bleomisien A_2 en B_2 onderskeidelik oor die 24 uur periode na behandeling. Plasma bleomisien A_2 en B_2 vlakke van 360.79 en 158.85 μg/ml onderskeidelik, is in monsters van kanker pasiënte met bleomisien IV behandel, gevind.

Die effekte van die verskillende middels op die migrasie van endoteelselle is bepaal deur ‘n aaneenlopende enkellaagselle te wond en dan die aantal selle wat weg beweeg vanaf die wond te bepaal. Die groei van endoteelselle is bepaal met behulp van selle wat met verskillende konsentrasies van die middels behandel is. Apoptose is ondersoek met behulp van weefselkleuring (hematoxylin en eosin), DNA fragmenteringsbepaling en akridien oranje kleuring.

Endoteelselle wat geinduseer is om kapillêragtige buise in ‘n kollageenjel te vorm, is gebruik vir die toetsing van die middels se effek op *in vitro* angiogenese. Die middels is dan ge-evalueer met betrekking tot die anti-tumor effek met die hemangioma muis-model.

Data het aangetoon dat die toetsmiddels endoteelselmigrasie onderdruk het, maar mytomycin C was die uitsondering hier en het nie die effek gehad nie. Al die middles het ‘n afname in lewensvatbare endoteelselle tot gevolg gehad en die afname was afhanklik van die dosis gebruik. Almal het ook endoteelselapoptose veroorsaak. Die middels het *in vitro* angiogenese onderdruk en het ook tumorontwikkeling *in vivo* tot ‘n meerder of minder mate onderdruk.

In die algemeen toon die resultate van hierdie studie dat daar nie ‘n noemenswaardige oorvloei van bleomisien in pasiënte met IB behandeling vir hemangiomas was nie en kan dus hier ‘n kleiner risiko vir die ontwikkeling van bleomisien-geinduseerde pulonêre fibrose wees.
Die studie het ook getoon dat die toetsmiddels angiogenese *in vitro* onderdruk asook hemangioma ontwikkeling in die *in vivo* muis-model. Hierdie opmerkings saam dui aan dat bleomisien hemangioma mag onderdruk deurdat angiogenese onderdruk word. Bykomstig, mitomycin C, 2-methoxyestradiol, taxol, vincristine, vinblastine, cochisine, nocodazole and cytochalisin D kan ook ‘n potensiële rol speel by die behandeling van hemangiomas en behoort met behulp van die *in vivo* hemangioma muis-model ondersoek word om die effektiewe dosisse en skedules te bepaal.

Sleutelwoorde: bleomisien, sitoskelet-ontwrigtingsagente, angiogenese, hemangioma, endoteelselle, selgroei, selmigrasie, vaskulêr endoteel groeifaktor, basiese fibroblast groeifaktor, poliomamiddel T onkogeen, vaskulêre tumor.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
bFGF	Basic fibroblast growth factor
BLM	Bleomycin
BME cells	Bovine microvascular endothelial cells
BBCE	Bovine brain capillary endothelial cells
Caspaces	Cysteinyl aspartate-specific proteases
C18 column	18-Caron reverse phase silica gel column
DdH2O	Deionised distilled water
MCF-7	Human breast carcinoma cell line
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DCS	Donor calf serum
EC	Endothelial cell
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
H&E	Hematoxylin and Eosin stain
HPLC	High performance liquid chromatography
HMEC-1	Human microvascular endothelial cells
IC_{50}	Inhibitory concentration for 50% of cells
IH	Infantile haemangiomas
MEM-α	Alpha-modified Eagle's minimum essential medium
MMC	Mitomycin C
mRNA	Messenger RNA
NaHS	Sodium heptane Sulphonate
ND	Not detected
PBS	Phosphate buffered saline
RIA	Radioimmunoassay
RNA	Ribonucleic Acid
rpm	Revolutions per minute
rRNA	Ribosomal RNA
tRNA	Transfer RNA
UV	Ultra violet

VBL	Vinblastine
VCR	Vincristine
VEGF	Vascular endothelial growth factor
vWF	von Willebrand Factor

CHAPTER 1

General Introduction

1.1. Motivation for the study

Haemangiomas are the most common tumours of infancy. [1-3] Although the incidence of haemangioma development has not been well documented, it is estimated that one in every ten children develops a haemangioma, most of which are on the head or neck. [1,2] The incidence of tumour development is increased to 22.9% for premature infants with a birth weight below 1 Kg. [2,3]

The pathophysiology of these vascular tumours is not well known. According to Pepper (1995), haemangioma development is associated with an imbalance of negative and positive regulators of angiogenesis. [4] Previously, light microscopic examination of haemangioma tissue demonstrated that the hallmark of the growing haemangioma was proliferating endothelial cells. [5,6] Furthermore, some of the growth factors which mediate the complex stages of angiogenesis, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), have been implicated in the pathogenesis of haemangiomas. [6,7] The recognition that excessive angiogenesis underlies haemangioma development offers an opportunity for the development of therapeutic strategies based on the inhibition of angiogenesis.

At present, the first-line treatment for haemangiomas is high-dose corticosteroids. [8,9] Steroid therapy for haemangiomas produces variable results. [1,2] Indeed according to Enjolras (1997), steroids dramatically regress haemangiomas in 30% of the patients; have little effect in 40%, and fail completely in the remaining 30%, with patients showing signs of worsening while on treatment. [1] Life-threatening haemangiomas that do not respond to corticosteroids are treated with interferon α. [8,9] Although success rates of approximately 80% have been reported with interferon α, there is a risk of developing irreversible neurotoxicity in haemangioma patients treated with the drug. [1]

Another pharmacological agent, bleomycin, has been cited as simple and adequate for the treatment of complicated cutaneous and massive symptomatic inoperable haemangiomas, without any severe complications. [10,11,12]

In studies undertaken at the University of Pretoria, South Africa, no major side effects were observed in haemangioma patients treated with intralesional bleomycin (IB), however, ulceration and flagellate pigmentation were observed in a small percentage of these patients (unpublished data).

The major complication of bleomycin treatment, pulmonary fibrosis, has been observed in cancer patients treated systemically with the drug. [13,14] It is not yet known whether IB therapy for haemangiomas carries the same degree of risk to the pulmonary vasculature as it does with intravenous administration for cancer chemotherapy. Determination of bleomycin spill-over levels is thus imperative to establish safety of use.

On the other hand, the mechanism by which bleomycin induces haemangioma regression is unknown, and there are concerns about the use of this chemotherapeutic drug to treat benign tumours. Kullendorf (1999) has attributed bleomycin's induction of haemangioma regression to the drug's possible sclerosing effect on vascular endothelium. [9] However, since haemangioma has been reported to be an angiogenic disease, it is plausible that bleomycin inhibits haemangioma growth by inhibiting angiogenesis. The elucidation of bleomycin's mechanism of action, and identification of other drugs with potential in the treatment of haemangiomas, represent important therapeutic objectives.

1. 2. Purpose of investigation

The initial aim of this study was to determine the degree of systemic circulatory spill-over of bleomycin in haemangioma patients who underwent IB therapy. Also, this study aimed to determine the effects of bleomycin on angiogenesis *in vitro* (in order to elucidate its mechanism of action in inducing haemangioma regression) and to compare bleomycin's effects on endothelial cells with those of drugs previously reported to inhibit aspects of angiogenesis, namely, mitomycin C, and cytoskeletal-disrupting drugs (2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole, and cytochalasin D). Lastly, the aim of this study was to test the effectiveness of bleomycin, mitomycin C, and various cytoskeletal-disrupting drugs *in vivo* in an animal model of haemangioma.

1. 3. Objectives

I. To develop a high performance liquid chromatographic method for the measurement of bleomycin in human plasma, and to use this method to determine bleomycin levels in haemangioma patients treated with intralesional bleomycin.

II. To determine the effects of bleomycin, mitomycin C, 2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole, and cytochalasin D on two of the key cell functions in the angiogenesis process, namely, endothelial cell migration and endothelial cell growth.

III. To determine the effects of test drugs (bleomycin, mitomycin C, 2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole and cytochalasin D) on endothelial cell apoptosis.

IV. To determine the effects of bleomycin and other test drugs on *in vitro* angiogenesis in a three-dimensional collagen gel model.

V. To induce vascular tumour development in a syngeneic mouse strain using pym T-immortalized endothelial cells and to determine the effects of bleomycin, mitomycin C, 2-methoxyestradiol, taxol, colchicine, and vinblastine on tumour development in this model.

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CHAPTER 2

Literature Review

I. Bleomycin

2.1. Chemistry of bleomycin

Bleomycin (BLM) is a generic name for a group of water-soluble glycopeptidic antibiotics isolated from the fermentation broth of *streptomyces verticillus*. [1-3] The antibiotics were initially extracted by a cation exchange resin process followed by separation on a sephadex G-25 column. The extraction yielded two biologically active copper-containing substances, bleomycins A and B. [1,3] These bleomycins were further separated chromatographically into six fractions of bleomycin A (A_{1-6}) and five fractions of bleomycin B (B_{1-5}). [3,4] In initial studies with Ehrlich carcinoma, the therapeutic effectiveness of the individual copper containing compounds was inferior to the results obtained with the mixture of BLM. [5,6] The reason for this apparent synergistic interaction among BLM fractions remains unclear. Pre-clinical and clinical development of BLMs focused upon a mixture comprising 55-70% A_2 and 25-30% B_2, and small quantities of a variety of other BLMs. [4,5] The current clinically used BLM, bleomycin sulphate USP (United States Pharmacopoeia), is formulated in this manner and is copper-free, due to early observations that the inclusion of copper induced significant phlebitis. [4]

Although the discovery of bleomycin was first reported in 1966, the exact chemical structure of the drug was only established a decade later. The structure was revised recently (fig 2.1).

2.2. Chemical Structure

A typical BLM molecule consists of 4 functional parts:

i) a metal binding region, which binds transition metals through several coordination links, and is also responsible for specific DNA sequence recognition; [6]

ii) the bithiazole part which is involved in DNA binding (the terminal amine parts of the bithiazole contribute to BLM's affinity for DNA); [6,7]

iii) a linker region, which is important in the efficiency of bleomycin's binding to DNA; [7]

iv) a carbohydrate domain whose function is still not clear, [7,8] it is likely that this domain participates in cellular uptake of bleomycin and metal-ion coordination. [8]

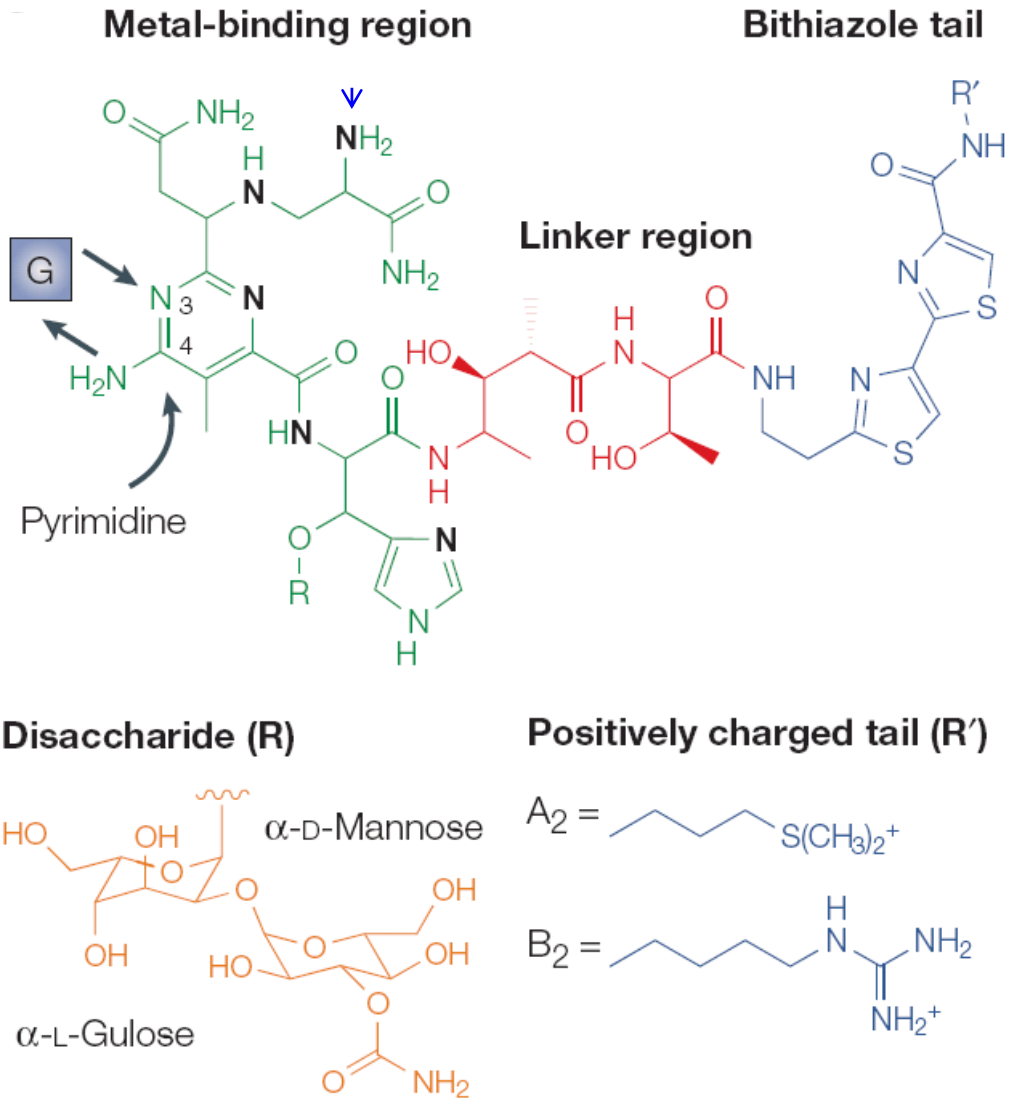

Figure 2.1. The chemical structure of bleomycin. The metal-binding domain is in green. The blue arrow points to the site of metabolic inactivation by bleomycin hydrolase. The nitrogen atoms that coordinate the metal are black. The N3- and N4- amino groups of the pyrimidine moiety of bleomycin (shown by 2 black arrows) are thought to define the specificity of DNA cleavage by binding to the N3- and N4- amino groups of guanine (G). The linker region is in red and the bithiazole tail in blue.

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Bleomycinic acid (BL) is the common structure of all bleomycins; it is a glycopeptide comprising 2 disaccharides (**R)** and 5 amino acids. The fractions, bleomycin A_2 and bleomycin B_2, differ at the c-terminus (positively charged tail of the Bithiazole moiety, **R′**). Bleomycin A_2 contains a dimethyl sulphonium propylamine linked to BL acid, while BLM B_2 contains an agmatine moiety (Fig 2.1). [6,8]

The c-terminal substituents (represented by **R′** in fig 2.1) appear to play a role in the binding of the bleomycin molecule to DNA. In previous studies removal of the c-terminal substituents resulted in diminished efficiency of DNA cleavage by bleomycin. [7,8]

2.3. Metal ion Coordination

The observation that the DNA degrading reaction by bleomycin exhibits an oxygen requirement and that the action of the drug can be terminated by chelating agents such as ethylenediaminetetraacetic acid (EDTA) led Horwitz and coworkers in 1976 to propose that the antibiotic requires a metal ion cofactor for its *in vivo* as well as *in vitro* activity. [6] Subsequently, bleomycin was shown to bind transition metals like Fe^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+} or Cu^{2+}. [6,7] Each of these ions can form a coordination complex with several amine groups of BLM.

When administered intravenously, bleomycins are given in metal-free form. Bleomycin rapidly binds to Cu(II) in blood plasma in an irreversible manner to form Bleomycin-Cu(II) or BLM-Cu(II). [7,8] It is believed that BLM-Cu(II) is the form in which bleomycin is transported into cells. Intracellularly, the BLM-Cu(II) can be reduced to bleomycin-Cu(I) and enter the nucleus or it can exchange with ferrous iron, Fe(II), to form bleomycin- Fe(II). Data from previous studies strongly suggest that a BLM-ferrous ion complex is the biologically active species. [6-8]

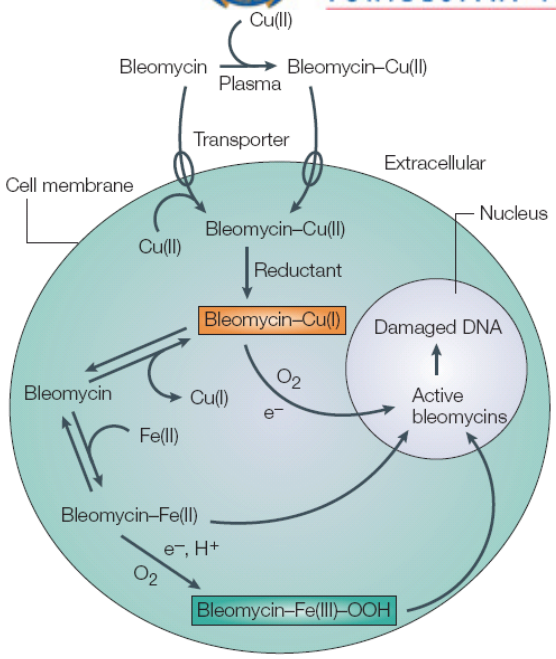

Figure 2.2. Proposed mechanism for the generation of 'activated bleomycin' *in vivo*.

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2.4. Mechanism of biological activity

The ability of bleomycin to bind and degrade DNA has been studied extensively, and consequently, DNA has for many years been accepted as the sole target of the drug's cytotoxic activity against neoplastic cells. Recently, RNA cleavage and inhibition of protein synthesis have been reported to constitute important additional elements of the mechanism of bleomycin activity *in vivo*. [7,9]

2.4.1. Effect of bleomycin on DNA

Horwitz demonstrated that bleomycin is capable of binding Fe(II) to yield bleomycin-Fe(II) or BLM-Fe(II). [5] Once formed, the BLM complex binds tightly to DNA with some evidence of intercalative interaction of the bithiazole moiety between guanosine-cytosine DNA base-pairs (fig 2.3). The oxidation of this complex by dioxygen to BLM-Fe(III)-OOH, the activated form of bleomycin, yields a radical. This radical is in turn responsible for DNA damage (fig 2.2; eqn 1 and 2). [8,9] Alternatively, activated bleomycin may also be generated in the cytosol and can then diffuse into the nucleus where it binds DNA (fig 2.2). [8]

$$\text{BLM Fe(II)} \xrightarrow{O_2,\ H^+,\ e^-} \text{BLMFe(III)-OOH} + \cdot\ \text{Radical} \quad (1)$$

$$\cdot\ \text{Radical} + \text{DNA} \longrightarrow \text{Damaged DNA} \quad (2)$$

The oxygen radicals produced by the bleomycin-iron complex bound to DNA primarily cause DNA single strand breaks, and to a lesser degree, double-strand breaks.

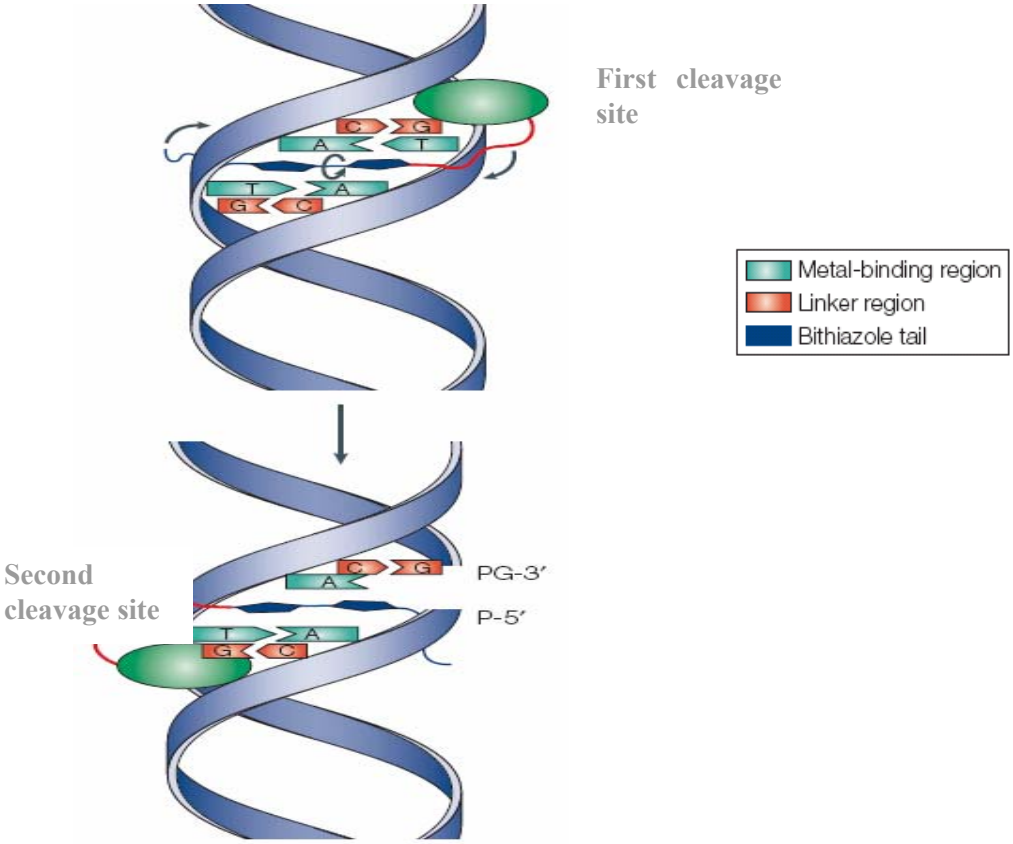

Figure 2.3. A model for dsDNA (double stranded DNA) cleavage by a single bleomycin molecule.

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It is assumed that every BLM molecule can produce up to 8-10 DNA strand breaks. BLM is able to make a second nucleophillic attack on the opposite strand, in a position nonsequence specific, +1 or –1 with respect to the first cleavage site. This nucleophillic attack on the second strand of DNA results in the generation of double-strand DNA breaks: one double strand break for 6 to 8 single-strand breaks on average. [6] The difficulty in repairing double-strand break lesions within DNA has been postulated to be the major source of BLM's toxicity.

2.4.2. Effect of bleomycin on RNA

Recently, RNA has been implicated as a potential target contributing to BLM's cytotoxicity. [10] It was observed in previous studies that BLM was able to cleave major classes of RNA (tRNA, mRNA, and rRNA).

Studies have also shown that BLM exhibits a strong cytotoxicity correlation with the antitumour agent onconase, which exerts antitumour activity through cleavage of RNA. In the Xenopus oocytes, BLM has been shown to mediate tRNA cleavage and consequently, to inhibit protein synthesis. [11] According to Abraham *et al*. (2003), RNA cleavage may constitute an important element of the mechanism of action of BLM. [12] However, the abundance and rapid turnover rate of RNA have meant that arguments for RNA as a primary target of bleomycin are not compelling.

2.4.3. Effect of bleomycin on proteins

Recent studies *in vitro* have demonstrated that bleomycin potentiates inhibition of protein synthesis. [11] However, this inhibition has been attributed to possible degradation of RNA. Nonetheless, very high concentrations of bleomycin are required to observe the effects of protein inhibition. [10]

There is a family of proteins that binds bleomycin and is highly specific for the drug. The proteins are known as bleomycin resistance proteins. They are found in microorganisms that produce bleomycin. However, these proteins have not been identified in mammalian cells. In microorganisms, the proteins form dimers that are located in the nucleus and inactivate BLM by forming stable complexes with the drug, which prevents the drug from reaching DNA. [11]

2.5. Metabolism of bleomycin

Bleomycin is hydrolyzed in the cytosol by the enzyme bleomycin hydrolase to deamidobleomycin, which is less active than bleomycin. In a previous study deamidobleomycin A_2 (which results from the hydrolysis of bleomycin A_2) was found to be 100-fold less potent in killing cultured murine L1210 cells than BLM A_2. [6]

The enzyme bleomycin hydrolase was initially identified in animal and yeast cell extracts, and was later found to be cytosolic. A cDNA encoding human bleomycin hydrolase was cloned in 1996. [6] Although the physiological role of bleomycin hydrolase is unknown, according to Lazo (1987), the level of the enzyme's activity in different tissues appears to play an important role in protecting tissues from bleomycin toxicity, and may define the spectrum of organs sensitive to the drug.

In a previous study, Umezawa *et al.* (1972) observed that the inactivation of bleomycin by the enzyme bleomycin hydrolase was very low in the lungs and skin, the two major sites of BLM–induced toxicity. [12] In marked contrast, greater rates of inactivation were observed in the liver, spleen, kidney, and bone marrow. A similarly designed study by Ohnuma *et al*. (1974), also found inactivation of BLM by bleomycin hydrolase to be low in the lungs and skin, and to be elevated in the liver, kidney, and spleen. [13] According to the authors, these studies showed bleomycin hydrolase activity to be elevated in those organs which are not clinically sensitive to BLM, and low in tissues affected by bleomycin. [6,12,13]

Although these earlier studies indicated that the prominent lung and skin toxicity of BLM could be related to the absence of bleomycin hydrolase, recent studies in yeast have revealed that over expression or deletion of the gene that codes for the enzyme does not affect BLM cytotoxicity. [12,13] Therefore, both the specificity of the enzyme against BLM, as well as its role in protecting cells from BLM remain questionable.

Another study cites cellular membrane transport as an important determinant of BLM sensitivity. [14] The cell membrane has previously been shown to limit bleomycin transport into cells. [14,15] According to reports, the drug's toxicity in certain cell types can be attributed to the presence of bleomycin transporters present on the surface of cell membranes. [15-17]

2.6. Side Effects

The most severe side effect of BLM is the induction of interstitial pneumonitis, which occurs in up to 46% of the patients. [18] Three percent of these patients later develop lung fibrosis.

The pathogenesis of lung fibrosis is not well understood, however, histological studies reveal that BLM induces damage to endothelial cells of the lung vasculature, which is followed by accumulation of inflammatory cells and collagen deposition in alveolar spaces, thus limiting oxygen exchange. Bleomycin-induced lung fibrosis represents a major draw back to the drug's clinical use.[19,20]

2.7. Clinical use

Bleomycins are effective against a variety of human neoplasms, particularly head and neck squamous carcinoma, Hodgkins and non-Hodgkins lymphomas, and testicular carcinoma. [15-17] Bleomycin has also been used for many years to treat viral warts. Additionally, a satisfactory therapeutic response in lymphatic malformations to local bleomycin injection has been reported since the 1970s. [18] More recently, bleomycin has been used successfully to treat vascular malformations and haemangiomas, and due to its efficacy and apparent lack of side effects, has prompted much clinical research into making it the drug of choice for haemangiomas. [21-23]

II. Haemangioma

2.8. Introduction

The term 'haemangioma' has been used to refer to various types of benign vascular neoplasms and malformations, which lead to much confusion, improper diagnosis and treatment of vascular lesions, and misdirected research efforts. [24] In an attempt to rationalize the nomenclature of vascular anomalies, Mulliken and Glowacki conducted a study on surgical biopsies from patients with vascular lesions and analysed these by histochemical, autoradiographic and electron microscopic techniques. [25] They then introduced a functional classification framework based on natural history, cellular turnover and histology of the various vascular lesions.

2.9. Nomenclature

Based on the work of Mulliken and Glowacki, vascular anomalies were classified into two major types: haemangiomas and vascular malformations. [24] A modification of this classification was accepted by the International Society for the Study of Vascular Anomalies (ISSVA) in 1996.

According to the modified classification, vascular anomalies can be divided into two major groups: proliferative and static (non-proliferative) lesions (table 2.1). [26]

Non-proliferative vascular lesions, also referred to as vascular malformations, are developmental anomalies or errors of morphogenesis and are further classified according to channel abnormality (*i.e.* arterial, venous, capillary, lymphatic or mixed malformations) or flow characteristics (*i.e*. high flow or low flow). Vascular malformations are thought to be present at birth, although they may not become evident or symptomatic until later in life. The lesions grow proportionately with the child and do not involute. The changes in the size of the lesions are related to haemodynamic changes, not cellular proliferation. [24-26]

Proliferative lesions include haemangiomas of infancy (referred to in this study as haemangiomas) and Kaposiform haemangioendothelioma. Newer subtypes of proliferative vascular tumours have recently been recognised. These tumours are fully formed at birth, and either involute rapidly and are termed rapidly involuting congenital haemangiomas (RICH), or fail to involute (even though they have features of haemangiomas) and are termed non-involuting congenital haemangiomas (NICH). [27]

Congenital haemangiomas can be distinguished from common haemangiomas of infancy in that they do not express GLUT1 (a glucose transporter that is widely distributed in fetal tissue; in adult tissue it is highly expressed in erythrocytes and endothelial cells of barrier tissues, such as the blood brain barrier). [23] Based on this ISSVA accepted classification, the term haemangioma should be restricted to a rapidly growing vascular tumour of infancy. [27]

Table 2.1: Classification of Vascular Anomalies

- **Vascular tumours**
  Infantile haemangioma, rapidly involuting congenital haemangioma, noninvoluting congenital haemangioma, tufted angioma, Kaposiform haemangioendothelioma
- **Vascular Malformations**
  - High-Flow
    Arteriovenous malformation (AVM)
    Arteriovenous fistula (AVF)
  - Low-Flow
    Venous malformation (VM)
    Lymphatic malformation (LM)
    Lymphatic-Venous Malformation (LVM)
    Capillary malformation ("port-wine stain")

High- or low-flow grouping were based on the flow dynamics within the lesion.

Haemangiomas are benign neoplasms of the vasculature. [28] The lesions, often referred to as infantile haemangiomas (IH), are considered to be the most common tumours of infancy. Haemangiomas can have deep, superficial, or mixed components. [29,30] The clinical appearance of haemangiomas varies with the degree of dermal involvement and the depth of the lesions. [31]

2.10. Natural History

Infantile haemangiomas (IH) have a unique natural history which is divided into three phases, the proliferative phase, the involuting phase and the involuted phase. [21] Most IH begin their growth in the first few weeks of life. [24] The proliferative phase is characterized by rapid growth of the lesion, while during the involuting phase there is a decline in growth, which is followed by the involuted phase or complete regression of the lesion. [24]

Light microscopic studies of haemangioma tissue have demonstrated that the hallmark of the growing haemangioma is a proliferation of endothelial cells, forming synctial masses, with or without lumina. [25] These studies have also revealed that in late stage, capillary-sized lumina may be seen to be lined by plump endothelial cells. [25]

The luminal surface of the endothelial cells exhibits thin projections, whereas the basal side has thicker, club-like projections. [24] Multilamination of the basement membrane has been cited as a pathological characteristic of the proliferative phase haemangioma. [25]

On the other hand, involuting haemangiomas show signs of vessel degradation. [24] During this phase, the haemangioma stabilizes, and appears to grow at the same rate as the child. Lumina contain endothelial cell remnants, often lined by only one or two endothelial cells. The involuted haemangioma is composed of thin-walled vessels that resemble normal capillaries. The basement membrane is still multi-laminated, although it is thin and disordered. [25]

2.11. Complications

Although most haemangiomas are symptomless, a subset of patients experience serious complications due to the location of the lesion or interference of the lesion with physiological function. [30-32] Complications associated with haemangiomas include airway obstruction, infection, ulceration, bleeding, pain and the development of congestive heart-failure, which is evident within the first few weeks of life in infants with hepatic haemangiomas. [31] On the face, haemangiomas can lead to disfigment. [31] As a result, such haemangioma patients require treatment. It is reported that without treatment, the mortality for hepatic haemangiomas is as high as 80%, and that early and aggressive treatment can lower mortality to approximately 20%. [1,31]

2.12. Pathophysiology

Growth factors, hormonal influences and mechanical influences have been postulated to underlie haemangioma development. [28] It is believed that a nascent haemangioma may result from endothelial cell proliferation secondary to increased levels of growth stimulating factors or decreased levels of normally present growth-inhibitory factors. [25,26] According to Mulliken and Young (1988), it is possible that tumour development can result from ‘an external stimulus to mitosis or a deficiency of an inhibitor, or an intrinsic biochemical defect in a localized endothelial cell population’. [25] It is also possible that all haemangiomas are not due to the same underlying defect.

According to Mulliken and Young (1988) the proliferating haemangioma is in many ways reminiscent of capillary proliferation as seen during wound healing and neovascularisation associated with tumour growth. [25]

Folkman (1995) and Pepper (1997) have described haemangioma growth as an example of an angiogenic disease, whereby an imbalance of normal vascular tissue turnover occurs and that the increased endothelial cell proliferation may be caused by abnormal levels of angiogenic stimulators or inhibitors. [33,34] Therefore, therapeutic strategies focused on angiogenesis inhibition may be effective in the treatment of these tumours.

2.13. Treatment

Various therapeutic modalities ranging from surgery to radiation therapy were originally employed in the treatment and management of haemangiomas. Understanding of the natural course of haemangiomas led to the development of newer therapeutic options, including medical (pharmacologic) therapies, which have become the mainstay in the treatment of haemangiomas, and are aimed at stopping progressive proliferation of the tumour or at accelerating involution. [35] Treatment modalities for haemangiomas have been classified by Zvulunov and Metzker (2002) based on their principal modes of action and are tabulated below (Table 2.2).

The current use of conventional surgery is limited and mainly used as apart of a multimodal approach for complicated haemangiomas (table 2.2). Although photocoagulation improves the appearance of haemangiomas, it has been reported to be associated with severe oedema. [35]

At present, complicated haemangiomas are treated initially with corticosteroids, systemically or intralesionally. [1,8] Systemic steroids have been used to treat ulcerated haemangiomas with variable efficacy. [33,35,36] Arterial embolization, surgery, and laser therapy have also been used in some cases. [35]

Table 2.2. Classification of therapeutic modalities by principal mode of action.

Medical	Surgical	Combined
Uncertain Mechanism:	Conventional excision	**Medical and surgical modalities:**
Steroids	Non selective vascular injury	Steroids & Laser
	Cryosurgery	Steroids & resection
Antiangiogenic Factors:	Ionizing radiation	
Interferon alpha	Super-frequency electromagnetic field	
Cytotoxic agents:	**Selective Photo coagulation:**	**Intralesional Injections:**
Cyclophosphamide	LASER ablation	Steroids
Vincristine	Intense pulsed light Photo-dynamic therapy	Bleomycin
Pingyangymycin		Pingyangymycin
Bleomycin		
Procoagulants:		
Tranexamic Acid		
Pentoxiphyllin		

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Potentially life-threatening haemangiomas that do not respond to corticosteroids can be treated with the angiogenesis inhibitor, interferon α. However, the risk of irreversible neurotoxicity with this form of treatment has been reported to be as high as 20% in haemangioma patients, and appears to be dose and duration dependent. [36]

Although cytotoxic chemotherapy is generally reserved for malignant disease, this modality has been used infrequently for biologically benign vascular tumours with serious complications (table 2.2). [35] Drugs used in this category include cyclophosphamide, vincristine, and pingyangymycin. However, none of these treatments has been studied systematically in the therapy of vascular tumours and none of these drugs has an established efficacy for the tumours. [35]

Another cytotoxic drug, bleomycin, was initially reported by Kullendorf to be an alternative treatment for complicated cutaneous and massive symptomatic inoperable haemangiomas. [21,22] Subsequent studies revealed that intralesional bleomycin induced accelerated resolution in haemangioma patients, without any severe complications. [21-23,37]
In a prospective study undertaken by the Pretoria Vascular Malformation Study Group, the

effectiveness of intralesional bleomycin (IB) treatment was evaluated. Of the 37 haemangioma patients treated with bleomycin, complete resolution or significant improvement was seen in 87% of patients (fig 2.4). [15] The study also showed an extremely low side-effect profile, with reported complications mainly including local pain and transient flu-like symptoms. [15] Ulceration and flagellate pigmentation were observed in a small percentage of patients (unpublished data). Levels of bleomycin in plasma samples of some of these patients are presented in chapter three of this thesis.

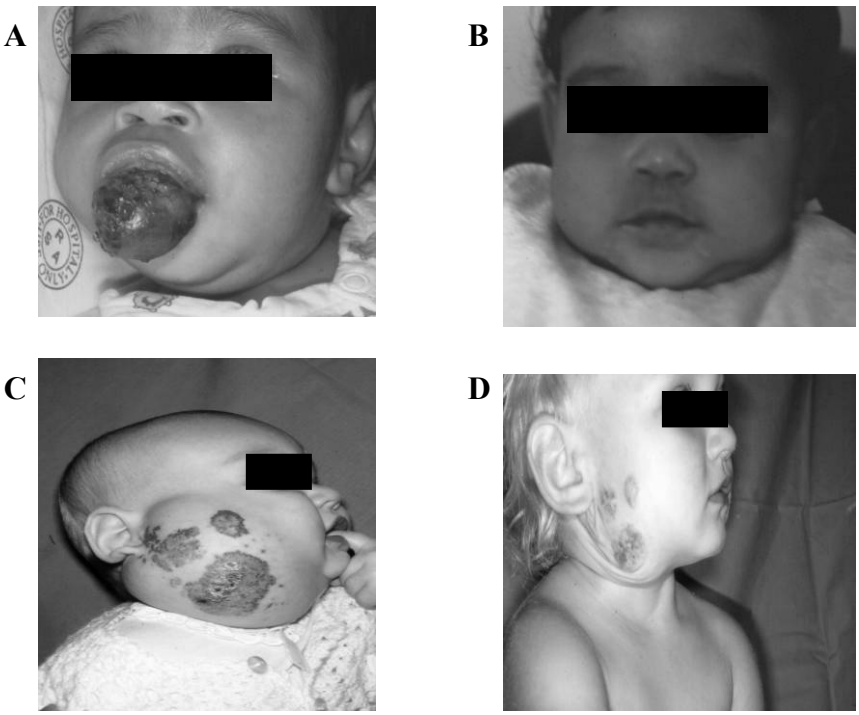
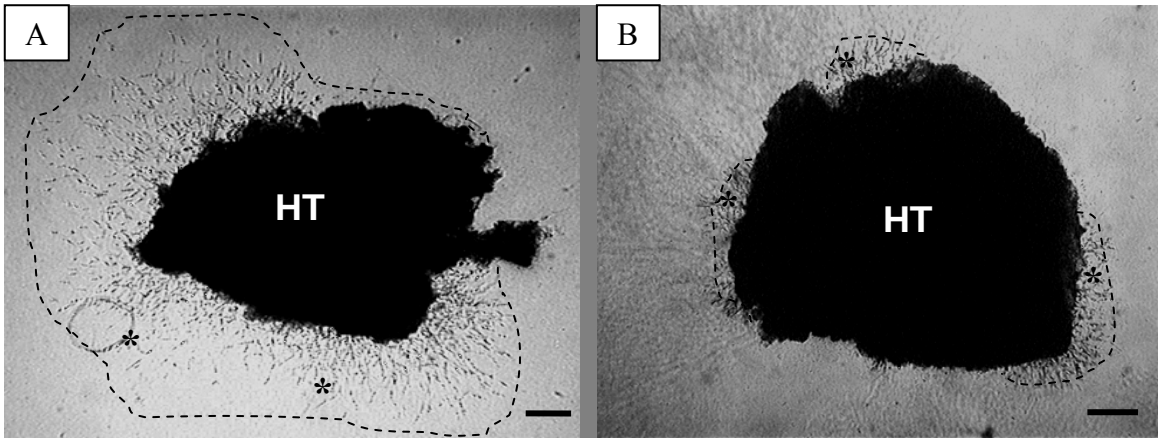


Figure 2.4. Haemangioma patients treated with intralesional bleomycin. **(A) -** a 2-month-old female infant with a histologically confirmed haemangioma originating from the superior alveolar ridge, with extra-oral protrusion of a rapidly enlarging lesion. **(B)** shows the result after 5 intralesional injections. **(C)** - a female infant with a facial proliferating haemangioma before IB, and **(D)** after 8 sessions of IB injections.

(Figure 2.4A was republished with kind permission from Springer Science and Business Media: Pediatr Surg Int, Intralesional bleomycin injection treatment for haemangiomas and congenital vascular malformations. Muir T et al., 19: 766-773, 2004; [15] *Permission conveyed through the Copyright Clearance Centre, Inc.).*

In another study conducted at the Cape Town Red Cross Children's hospital, following the treatment of 30 haemangioma patients with intralesional bleomycin, a response rate of 75 to 100% was attained in 73% of the patients; a response rate of 50 to 75% was reported for the rest of the patients. [37] Despite these impressive results, the mechanism of action of bleomycin in haemangiomas remains unknown. [38]

Studies were undertaken under the supervision of Dr P.F. Davies in the initial stages of this PhD to determine the effects of bleomycin on cultured human haemangioma biopsies. From these studies, vessel-like structures emanating from the surface of cultured tumour biopsies in both control and BLM-treated cultures were observed. However, fewer vessel-like structures were observed in tissue fragments treated with BLM (fig. 2.4B). Previously, work conducted in the same laboratory using this model showed that Von Willebrand factor (vWF) and CD31 were localized to the vessel-like outgrowths, confirming that these were neovessels. [39]



Bar = 250 µm

Figure 2.5. Haemangioma Tissue (HT) cultured in fibrin gel. Haemangioma Tissue gave rise to an array of microvessels (asterisks) emanating from its surface (extent indicated by dotted line). A - Control; B - BLM-treated tissue.

The findings from these studies on the effects of bleomycin on human haemangioma *in vitro* therefore indicated that bleomycin may inhibit haemangioma growth in patients by inhibiting angiogenesis.

III. The Angiogenesis Concept

2.14. Introduction

Angiogenesis is the formation of new capillary blood vessels from pre-existing vessels. [40] The process of angiogenesis involves a series of complex and sequential events previously described by Pepper (1995; 2001) and Papetti and Herman (2002): [34,41,42] The process begins with the removal of periendothelial cells from the endothelium and vessel destabilization by angiopoietin-2 (Ang-2). [42] Vessel hyperpermeability, induced by vascular endothelial growth factor, allows for the extravasation of fibrinogen from the circulation, with the subsequent formation of a fibrin matrix. [41,42] Degradation of the basement membrane and other ECM components is induced by a cohort of extracellular proteases and their inhibitors. According to Pepper (2001), most of these proteolytic enzymes belong to one of two families: serine proteases (in particular the plasminogen activator-plasmin system), and the matrix metalloproteinases (MMPs). [41,43]

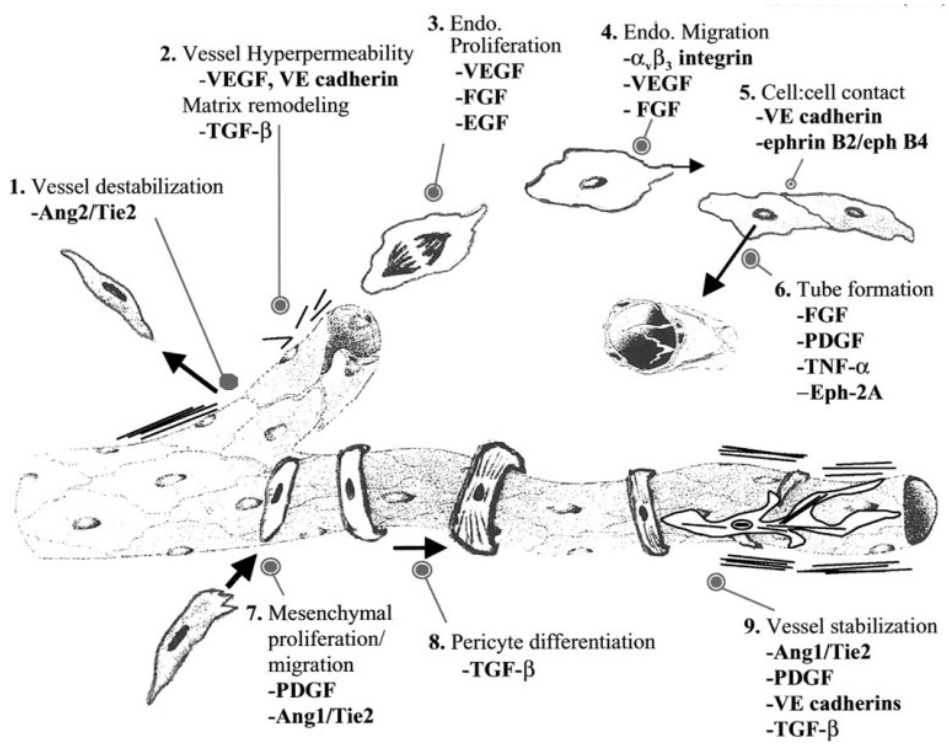


Figure 2.6. Schematic diagram illustrating the process of angiogenesis. See text for details.

Reproduced with permission from Papetti M and Herman IM. Mechanisms of Normal and Tumor-Derived Angiogenesis. Am J Physiol Cell Physiol, 2002; 282: C947-70. Permission conveyed through the Copyright Clearance Centre, Inc.) [42]

Following the breakdown of the basement membrane, endothelial cells migrate and proliferate in the direction of the angiogenic stimulus (through the remodelled matrix). Endothelial cells then form a microvessel sprout. [42]

This is followed by branching of the newly formed microvessel sprouts and formation of arcades by fusion with neighbouring new microvessel sprouts through which blood flow can begin. [41,42]

The establishment of endothelial cell quiescence, strengthening of cell-cell contacts and the elaboration of a new matrix, all serve to stabilise the newly formed vessel. [42,44] The process of angiogenesis is summarised in fig 2.6, and the role of cytokines and growth factors depicted in the diagram are tabulated in appendix I. The role of these cytokines and growth factors in pathological angiogenesis are tabulated in appendix II.

2.15. Growth factors in angiogenesis

A number of growth factors, cytokines and their receptors (listed in appendix I) have been reported to mediate the complex stages of angiogenesis including endothelial cell migration, proliferation, tube formation, and stabilization of developing vessels. [40] Two of the most potent and highly characterized of the angiogenic growth factors, namely, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), were employed in this study, and are thus discussed in this chapter.

2.15.1. Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF, initially known as vascular permeability factor or VPF) is a glycosylated protein with a C-terminal heparin-binding domain. [44] The family consists of VEGF, -A, -B, -C, -D, and Platelet derived growth factor (PDGF). [44,45] The most characterized angiogenic growth factor in this family is vascular endothelial growth factor A (which will from now on be referred to in this study as VEGF). [46] Alternative splicing of a single gene generates six isoforms of VEGF composed of 121, 145, 165, 183, 189, and 206 amino acids, although VEGF165 is the most commonly expressed isoform. [44]

The receptors for VEGF are expressed on vascular endothelial cell surfaces. [44-46] These receptors are fms-like tyrosine kinase-1 (Flt-1) or VEGFR-1, foetal liver kinase-1 (Flk-1) or VEGFR-2, fms-like tyrosine-kinase-4 (Flt-4) or VEGFR-3, and neuropilin-1 and -2. [45,46]

Table 2.3. Receptors to the VEGF ligand and their biologic effects.

VEGF Family Members	Receptor	Function
VEGF-A	VEGFR-1, VEGFR-2, neuropilin-1	Angiogenesis, vascular maintenance
VEGF-B	VEGFR-1	Not established
VEGF-C	VEGFR-2, VEGFR-3	Lymphangiogenesis
VEGF-D	VEGFR-2, VEGFR-3	Lymphangiogenesis

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Vascular endothelial growth factor is produced by many cell types including vascular smooth muscle cells, lung alveolar epithelial cells, macrophages, platelets as well as a wide variety of tumour cells. [42] It is a paracrine factor and an important mediator of vasculogenesis (the formation of new blood vessels from mesenchymal precursor cells) and angiogenesis. [42]

In endothelial cells, VEGF mediates mitogenic signals by activating VEGFR-1 and-2. [43] However, compared with VEGFR-1, VEGFR-2 has less affinity for VEGF, even though it presents a greater signalling activity. [45] The mitogenic activity in endothelial cells is mediated mainly by VEGFR-2. [42,45] Additionally, VEGFR-2 mediates cell migration and vascular permeability in response to VEGF, whereas VEGFR-1 has a weak or undetectable response. [45,47] The signalling pathways activated by VEGF and some of its physiological roles in angiogenesis are shown in fig 2.7.

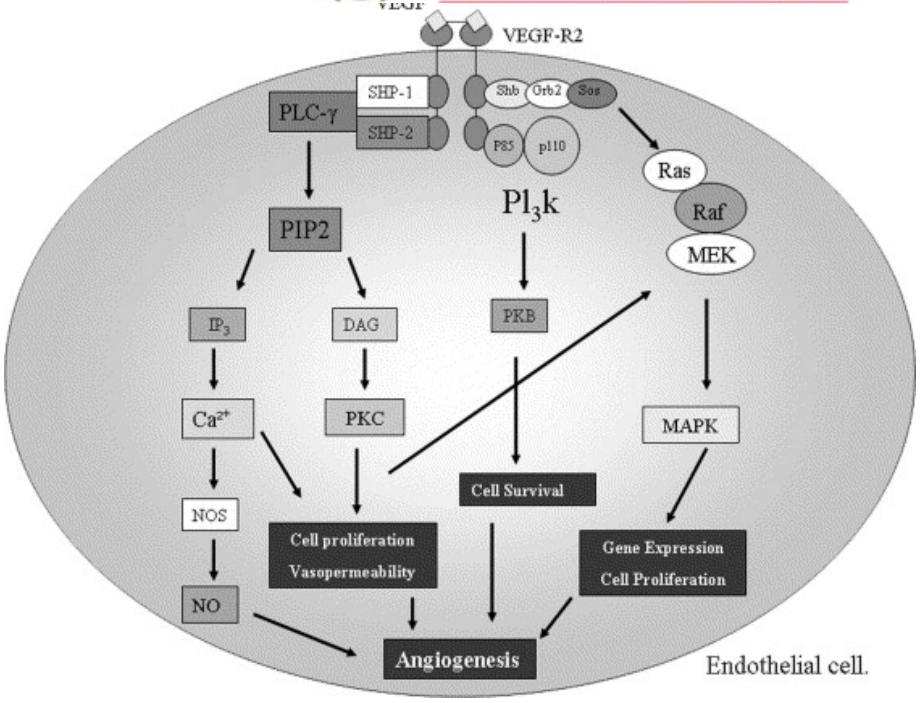

Figure 2.7. Signalling pathways of vascular endothelial growth factor.

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Upon binding of VEGF to VEGF-R2, the receptor is phosphorylated, allowing the receptor to associate with and activate a range of signalling molecules, including phosphatidylinositol 3-kinase (PI3K), Shc, Grb2, and the phosphatases SHP-1 and SHP-2 (fig 2.7). VEGF receptor activation can also induce activation of the MAPK cascade via Raf stimulation leading to gene expression and cell proliferation, while activation of PI3K leads to PKB activation and cell survival, and activation of PLC-g leads to cell proliferation, vasopermeability, and angiogenesis. [45]

In vitro, VEGF promotes neovessel formation in three-dimensional models of angiogenesis; it was also reported to promote the formation of vessel sprouts from rat aortic rings embedded in a collagen gel. [45,47] VEGF also elicits a pronounced angiogenic response in a variety of *ex vivo* and *in vivo* models, including the chick chorioallantoic membrane (CAM) assay, the rabbit cornea, and the matrigel plug in mice. [46]

A wide variety of human and animal tissues express low levels of VEGF, but high levels of the ligand are produced when angiogenesis is required, such as in foetal tissue, the placenta, the corpus luteum, during inflammation, as well as in a vast majority of human tumours. [46]

2.15.2. Basic fibroblast growth factor

Basic fibroblast growth factor (bFGF or FGF-2) is an 18 kDa molecule present in various sources including EC and tumour cells. [48] It has been reported to play an important role in angiogenesis, especially in synergy with VEGF. [48] It exerts its action by binding to tyrosine kinase receptors FGFR-1, -2, -3 and -4. Like VEGF, bFGF induces processes in endothelial cells *in vitro* that are critical for angiogenesis, such as endothelial cell proliferation and migration as well as endothelial cell production of plasminogen activator and collagenase. In addition, bFGF causes endothelial cells to form tube-like structures in three-dimensional collagen matrices (Fig 2.8). [48,49]

However, unlike VEGF, which is mitogenic primarily for endothelial cells, bFGF stimulates proliferation of most, if not all cells derived from the embryonic mesoderm and neuroectoderm, including pericytes, fibroblasts, myoblasts, chondrocytes, and osteoblasts. [47,48]

According to Presta *et al*. (2000), bFGF does not appear to play a major role in physiologic angiogenesis *in vivo* (Fig 2.8), but may be released upon cell disruption by an injury where it is deposited in the extracellular matrix. [47] The growth factor might thus have a role in local reparative angiogenesis following tissue injury. Indeed, mice deficient in fibroblast growth factors display mild defects in wound healing. [48]

In vitro, basic fibroblast growth factor was shown to play induce endothelial cells to recapitulate several aspects of the *in vivo* angiogenesis process, including the modulation of the production of proteases involved in the degradation of the basement membrane, endothelial cell proliferation, migration, integrin and cadherin receptor expression (Fig 2.8). [48] In addition, several experiments also implicate bFGF in the pathogenesis of haemangioma. [25,39]

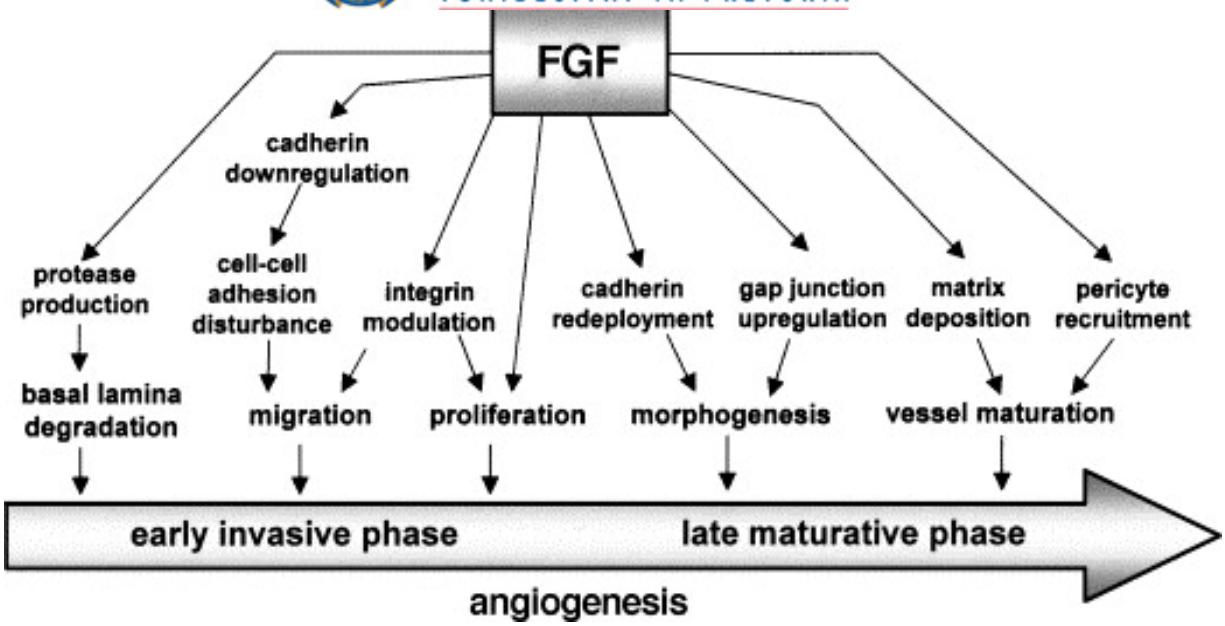

Figure 2.8. Schematic representation of the effects of bFGF in endothelial cells that contribute to the acquisition of the angiogenic phenotype *in vitro* and to neovascularization *in vivo*. *Reprinted from* Cytokine & Growth Factor Reviews 16: *159-178, Presta M, Dell'Era P, Mitola S, Moroni E, Ronca R, Rusnati M.* Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *2005, with permission from Elsevier.*

Both VEGF and bFGF have been shown to promote endothelial cell survival and to suppress apoptosis. [48,50]

2.16. Apoptosis

Suppression of apoptosis has been cited as important for the process of angiogenesis. According to Chavakis and Dimmeler (2002), *in vitro* studies have shown that growth factor deprivation leads to programmed cell death of endothelial cells. [50] In their review, the authors use the terms apoptosis and programmed cell death synonymously. Therefore, in order to continue the discussion of apoptosis, it is necessary to have a complete understanding of the terminology and definitions used in cell death.

Various studies have classified cell death into two categories, programmed cell death and necrosis. [51,52] Programmed cell death refers to any form of death a cell may undergo that is mediated by an intracellular program. Originally, programmed cell death and apoptosis were used interchangeably. [51,53] However, it later became evident that cells could undergo programmed cell death without the characteristic morphological changes observed in apoptosis. [54-55]

Recently, other models of programmed cell death (PCD) were proposed (fig 2.9) and these include (in addition to apoptosis): [51,52,54,55,56,57]

- **Entosis**, a form of cell death induced by cell detachment from the extracellular matrix, and which involves the engulfing of detached cells by other cells (cell-in-cell invasion).
- **Paraptosis**, which involves cytoplasmic vacuolation and mitochondrial swelling (in the absence of caspase activation);
- **Mitotic catastrophe**, which is a default pathway after mitotic failure;
- **Slow cell death**, a form of PCD used to describe the delayed type of death that occurs if caspases are inhibited or absent; and
- **Autophagy**, a form of PCD characterized by sequestration of cytoplasmic organelles and their subsequent degradation by the cell's lysosomes.

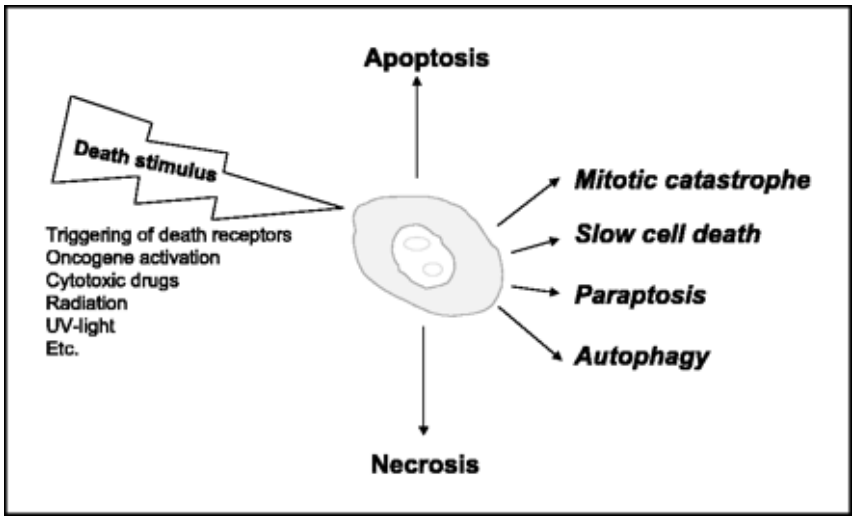

Figure 2.9. Models of cell death. See text for details.

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Necrosis, on the other hand, is a type of cell death initially regarded as the counterpart of programmed cell death. Necrosis is characterised by cellular swelling, often accompanied by chromatin condensation and eventually leading to cellular and nuclear lysis with subsequent inflammation. [58,59] Another important descriptive term in cell death is oncosis. Majno and Joris (1995) proposed the use of the term oncosis for designating any cell death characterised by swelling (instead of necrosis), while the term necrosis refers to features which appear after the cell has died (fig 2.10). [51,60]

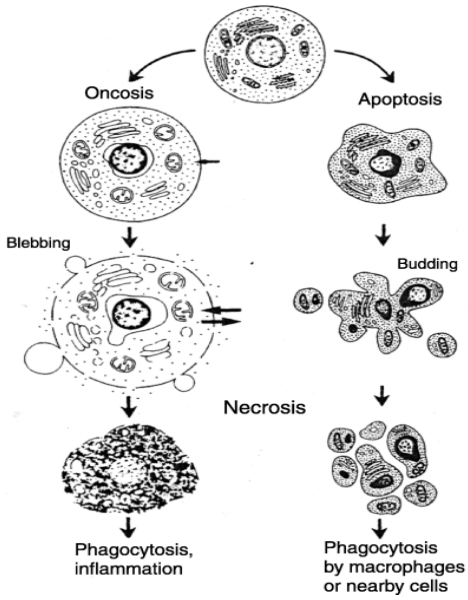

Figure 2.10. Apoptosis and oncosis.
Majno G, Joris I. Apoptosis, oncosis and necrosis. An overview of cell death. Am J Path 1995; 146:3-5. [61]

Reports have cited a balance between cell growth and cell death (apoptosis and necrosis) as being crucial for the maintenance of homeostasis of the vascular endothelial cell population, [61] however, the role of necrosis or of other forms of PCD in the maintenance of such a balance has not been explored. In contrast, the importance of apoptosis in angiogenesis has been widely cited. [50,61] Also, increased apoptosis has been associated with the involuted phase of the haemangioma life cycle. [2,5,6] Indeed, excessive endothelial cell proliferation which is not balanced by apoptosis is one of characteristics of the growing haemangioma.

Apoptosis is a form of programmed cell death marked by cellular shrinkage, chromatin condensation, and budding of the plasma membrane. [60-64] Apoptosis is generally the result of the activation of a subset of caspase proteases. [60,61,63]

According to Folkman (2003) apoptosis induction in microvascular endothelial cells can lead to regression of tumour tissue. [64] Thus various agents with the ability to induce apoptosis may have therapeutic potential as antiangiogenic drugs. Recognition of the potential therapeutic benefits of controlling pathologic angiogenesis has lead to a search for new targeted antiangiogenic agents and re-evaluation of existing chemotherapeutic drugs. [65] Mitomycin C, a chemotherapeutic drug previously reported to induce apoptosis in a number of cancer cell lines and to inhibit endothelial cell proliferation, was investigated in this study.

2.17. Mitomycin C

Mitomycins are a group of antibiotics isolated from *Streptomyces caespitosus*. [66] Of the mitomycins isolated, mitomycin C has proven to be superior in antitumour potency, and is therefore the only one currently in clinical use. [66] Clinical application of mitomycin C includes adenocarcinomas of the stomach, colon and pancreas. [67] For the treatment of these neoplasms, the drug constitutes an essential basis of combination regimens such as MOB (mitomycin C, vincristine, and bleomycin).

Mitomycin C is a DNA-alkylating agent which is activated *in vivo*. [66] After activation, cytotoxic activity can be observed owing to covalent binding and cross-linking of DNA. [66] Mitomycin C was also reported to inhibit the proliferation of cultured human dermal microvascular endothelial cells. [68]

The effects of various cytoskeletal-disrupting agents, previously reported to inhibit aspects of angiogenesis were also investigated. Cytoskeletal components affected by these agents are discussed below, and the effects of these agents on aspects of angiogenesis are summarized in table 2.3.

2.18. The Cell Cytoskeleton

The cytoskeleton is a dynamic 3-D scaffold in the cytoplasm of a cell. It is essentially constituted by three components: microfilaments, intermediate filaments, and microtubules. [69] Both microfilaments and microtubules play important roles in mitosis, cell signalling and motility, and are targets for a number of antitumour drugs. [69-72] These two cytoskeletal filaments are thus discussed in detail.

2.18.1. Microfilaments

Microfilaments are fine thread-like protein fibres 3-6 nm in diameter. [69,70] They are responsible for cellular movements including contraction, gliding and cytokinesis.

Microfilaments are composed predominantly of the contractile protein actin and are thus also referred to as actin filaments. [70] Actin filaments have polarity, the plus (+) end is the end that is opposite the cleft that holds the ATP molecule, and the minus (-) end is the opposite end (fig 2.11A,C). [70] Growth and polymerization is more rapid at the plus end, while depolymerisation predominates at the minus end. [70]

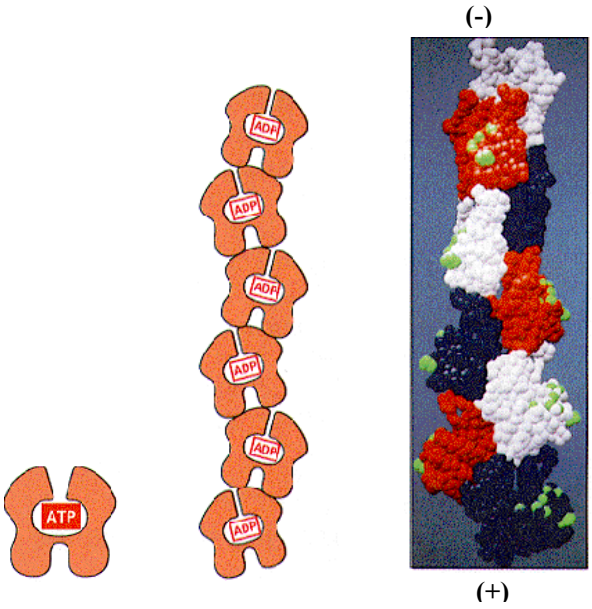

Figure 2.11. The structure of actin. (A) – an actin monomer, G-actin; (B) – a growing actin protofilament formed by multiple monomers; (C) – the actin filament, F-actin.
http:users.rcn.com/jkimball.ma.ultranet/BiologyPages/C/**Cytoskeleton**.html

The actin monomer (fig 2.11 A), termed G-actin, forms a dimer by combining with another actin monomer, however, the binding is weak. Formation of a trimer stabilizes the complex of actin monomers and serves as a site for nucleation, the initial stage of polymer formation (fig 2.11 B). G-actin then forms F-actin, the filament (fig 2.11 C), through elongation (addition of molecules of actin to form a long helical polymer). Above a critical concentration of G-actin, the molecules polymerize. [70]

Elongation of the polymer occurs at each end by reversible, non-covalent addition of G-actin subunits. The actin filament exhibits complex polymerization dynamics that utilize energy provided by the hydrolysis of adenosine nucleotide triphosphate (ATP). The hydrolysis of ATP during F-actin polymerization creates dynamics which are referred to as non-equilibrium dynamics, in which the addition of actin occurs at the plus end, with loss occurring at the minus end. [70,73]

Studies have shown that the ratio of polymerized actin to soluble actin is reduced in transformed cells than in non-transformed cells. [69] Indeed, it was shown more than three decades ago that the actin cytoskeleton is substantially modified in transformed cells. [73] Furthermore, a study showed transformed cells to be more sensitive to cytochalasin B, an actin filament-disrupting agent, than nontransformed cells. [73] Cytochalasin D, another microfilament-disrupting agent, binds to the plus end of F-actin and prevents further addition of G-actin, thus preventing polymerization, but not depolymerization. [71]

2.18.2. Microtubules

Microtubules are cylindrical tubes which are 20-25 nm in diameter. They are composed of tubulin subunits, which are termed alpha and beta. [69,71] Microtubules are involved in locomotion, they determine cell shape, and they provide a set of tracks for cell organelles and vesicles to move on. [74] They also form spindle fibres for separating chromosomes during mitosis. [74]

In the cell itself, microtubules are formed in an area near the nucleus, the microtubule organising centre (MTOC). Microtubules are polar, with a plus end (fast growing) and a minus end (slow growing), usually the anchor point in the MTOC. [69,71] The first stage of microtubule formation is called nucleation. During nucleation, an alpha tubulin molecule and a beta tubulin molecule join to form a heterodimer. Two or more heterodimers then attach to other dimers to form oligomers which elongate to form polymers called protofilaments. [71]

Similar to microfilaments, microtubules exhibit complex polymerization dynamics, however, microtubule dynamics utilize energy provided by the hydrolysis of guanosine nucleotide triphosphate (GTP).

The hydrolysis of GTP occurring during microtubule polymerization creates two forms of dynamic behaviour in cells: i) dynamic instability, during which microtubule ends switch between episodes of prolonged growing and shortening, with the plus end showing more instability than the minus end. ii) tread-milling, which occurs due to differences in the critical subunit concentrations at opposite ends and consists of net growing at the plus end and net shortening at the minus end.

Microtubule dynamics have been cited as being important in multiple processes, including mitosis. When cells enter mitosis, the microtubule network is dismantled, and a bipolar spindle shaped array of microtubules is built. This microtubule array attaches to chromosomes and moves them to the two spindle poles. According to reports, microtubule dynamics are slow in interphase cells, but increase 20-fold at mitosis. [71,73] Different drugs affect microtubule dynamics: colchicine and nocodazole inhibit polymerization by binding to tubulin and preventing its addition to the plus end. The vinca alkaloids lead to microtubule depolymerisation, while taxol stabilizes the microtubule by binding to a polymer. [73-76]

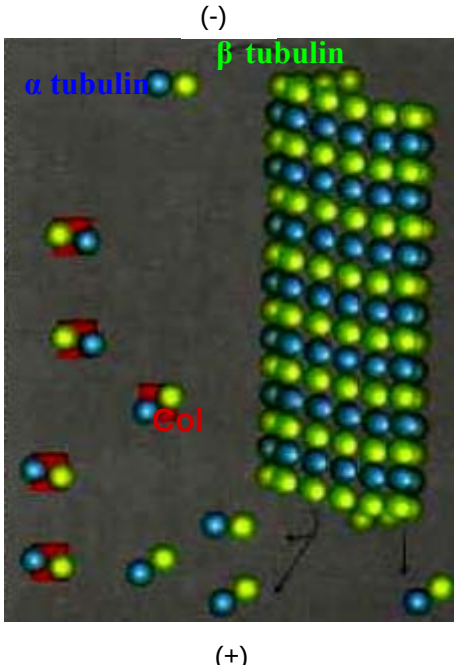

Figure 2.12. Longitudinal section through a microtubule. Colchicine (red) prevents polymerization by binding to tubulin heterodimers and thus preventing their addition to the plus end. http:users.rcn.com/jkimball.ma.ultranet/BiologyPages/C/**Cytoskeleton**.html

According to Pasquier *et al*. (2006), the cellular effects of microtubule-disrupting agents result in anti-angiogenesis through the inhibition of endothelial cell migration, endothelial cell proliferation and differentiation as well as extracellular matrix (ECM) and basement membrane (BM) degradation. [74]

The impaired mobilization and reduced viability of circulating endothelial progenitor cells (CEPs), though not well-studied (and thus represented by a dotted arrow in fig 2.13), has also been implicated as a contributing factor in the anti-angiogenic effects of these agents.

Recently, the mobilization of CEPs was reported to promote tumour angiogenesis. [74] Khan *et al*. (2006) reported that haemangiomas were composed of CEPs which differentiate into mature endothelial cells that comprise a 'major compartment of the tumour'. [77]

Generally, the effects of antiangiogenic agents have been classified either as direct effects when these agents act on endothelial cells or indirect effects when they act on tumour cells. [78] Similarly, microtubule-disrupting agents are classified as direct or indirect inhibitors of angiogenesis (table 2.4). [74] The effects of drugs that affect microfilament dynamics have not been well-documented. The various antiangiogenic effects of microtubule-disrupting drugs employed in this study, and that of the actin-disrupting drug, cytochalasin D, are outlined in table 2.4, and these drugs are discussed briefly below.

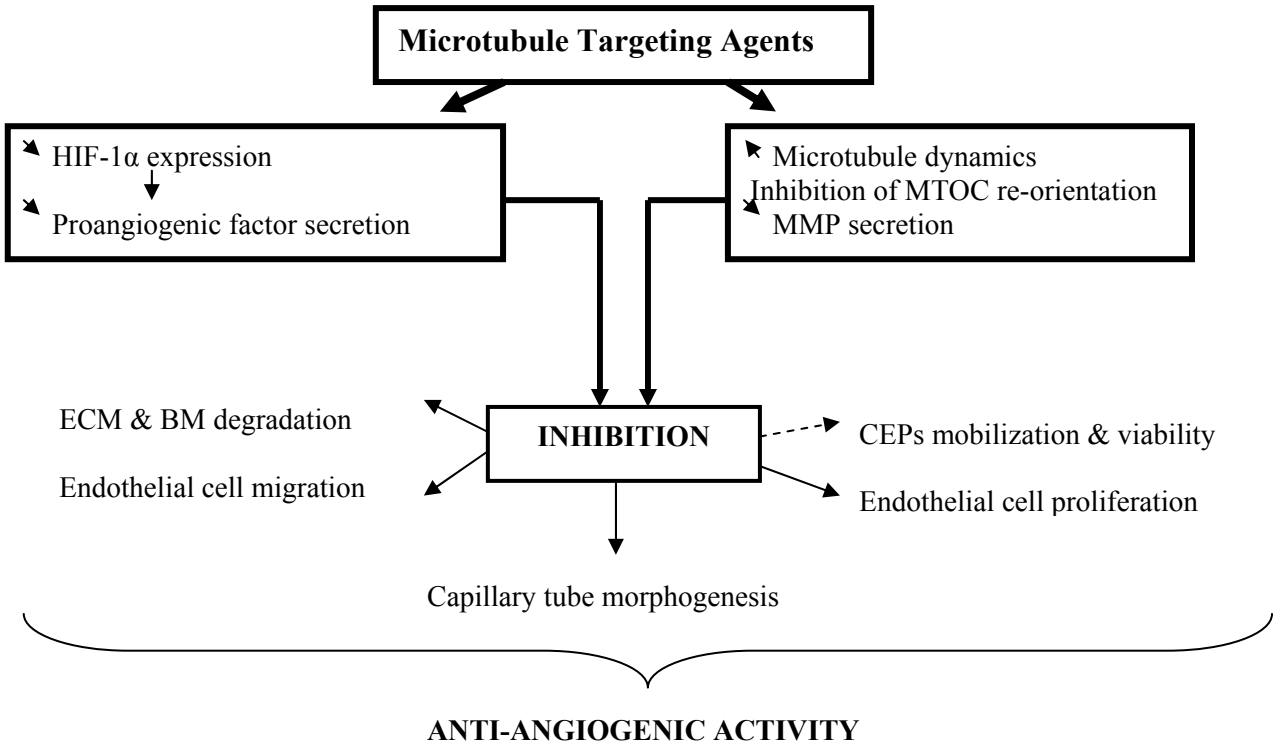

Figure 2.13. Mechanisms involved in the anti-angiogenic effects of microtubule-disrupting drugs. See text for details. HIF-1- ; MMP- matrix metalloproteinase; MTOC- microtubule organising centre; ECM-extracellular matrix; BM-basement membrane; CEPs- circulating endothelial progenitor cells.

Reproduced from Pasquier E, Honore S, and Braguer D. Microtubule-targeting agents in angiogenesis: where do we stand. Drug Resistance Updates. 2006; 9:74-86. Copyright (2006), with permission from Elsevier. [76]

2.18.3. Colchicine

Colchicine is an alkaloid produced by the colchicum species *e.g.* C. automnale. Colchicine is widely used in the treatment of gout, and is often used in the laboratory to induce mitotic arrest in various cells. [79,80] *In vitro*, it binds to tubulin dimers, and inhibits their assembly into microtubules. [79] The colchicine binding site on tubulin is believed to be located on ß-tubulin. A range of unrelated microtubule inhibitors bind to tubulin at or near the colchicine site. [71]

2.18.4. 2-Methoxyestradiol

2-Methoxyestradiol (2-ME) is an endogenous metabolite of 17 ß-estradiol derived from O-methylation of 2-hydroxyestradiol and a potent inhibitor of endothelial cell growth and migration, and is extremely weak in binding to estrogen receptors. [81,82] 2-ME is the most potent endogenous inhibitor of tubulin polymerization yet described. [79,80] It is a weak competitive inhibitor of the binding of colchicine to tubulin, and has been shown to arrest growth in a variety of tumour cell lines, and to induce apoptosis in these cell lines. 2-ME has also been shown to inhibit tumour growth *in vivo*. [79,82]

2.18.5. Vincristine and Vinblastine

Vincristine and vinblastine are plant alkaloids that inhibit microtubule assembly by binding tubulin and inducing self-association in spiral aggregates in a reaction that appears to be regulated by the C-terminus of ß–tubulin. [76] They bind to tubulin at a site distinct from the colchicine-binding site. [76]

Vinblastine inhibits tubulin dependent GTP hydrolysis and stabilizes the microtubule, in particular the plus end, and it depolymerises microtubules at the minus end. [71,83] Vinca alkaloids block mitotic spindle formation and induce cell-cycle arrest in G2/M. [83] In addition, vinca alkaloids induce apoptosis in several tumour cell lines. [83]

2.18.6. Nocodazole

Nocodazole is a benzimidazole compound that inhibits microtubule assembly in a dose-dependent manner. [84] It binds to ß-tubulin and prevents formation of 1 or 2 interchain disulfide linkages, thus inhibiting microtubule dynamics.

This leads to disruption of mitotic spindle function and the arrest of the cell cycle at G2/M transition. Nocodazole induces apoptosis in several tumour cell lines. [84]

2.18.7. Paclitaxel

Paclitaxel, commonly known as taxol, is derived from the bark of the western yew tree. It binds to the N-terminal region of ß-tubulin and promotes the formation of highly stable microtubules; the microtubules resist depolymerisation. [85,86] This prevents normal cell division and arrests the cell cycle at G2/M transition. [85] Paclitaxel has antitumour activity against a number of cell lines, and is effective against ovarian, breast, lung and head and neck carcinomas. [87-89]

2.18.8. Cytochalasin D

Cytochalasins are fungal toxins, and are the best studied and most widely used agents that act on actin. [84] Cytochalasin D, one of the cell permeable fungal toxins, is a potent inhibitor of actin polymerization. It activates the p53 pathway and arrests the cell cycle at G1/S transition. [84]

Table 2.4. Cellular effects of cytoskeletal-disrupting agents associated with their anti-angiogenic activity.

Drug	Model	Antiangiogenic effects	References
		Direct effects	
Nocodazole	Bovine capillary endothelial cells	Inhibition of cell growth.	Ingber *et al.* (1995) [84]
Paclitaxel	HUVEC HMEC-1	Initiation, without completion, of the mitochondrial apoptotic pathway in vitro, leading to a slowing down of the cell cycle.	Pasquier *et al.* (2004) [90]
Paclitaxel	HUVEC HMEC-1	Increase in interphase microtubule dynamics *in vitro*.	Pasquier *et al.* (2005) [91] Pourroy *et al.* (2006) [92]
Paclitaxel	HUVEC HMVEC-L HMVEC-D	Increase in the drug cellular uptake in human endothelial cells as compared with fibroblasts and tumour cells *in vitro*.	Merchan *et al.* (2005) [93]
Vinblastine	SCID mice bearing murine or human breast cancer	Rapid decline in CEPs viability *in vivo*.	Shaked *et al.* (2005) [94]
Cytochalasin D	Bovine capillary endothelial cells	Inhibition of cell growth.	Ingber *et al.* (1995) [84]
		Indirect effects	
Paclitaxel Vincristine	Nude mice bearing murine breast cancer; human leukemia cell lines	VEGF down-regulation *in vitro* (even in drug resistant cells) and *in vivo*.	Lau *et al.* (1999) [95] Avramis *et al.* (2001) [96]
2-ME2 Vincristine Vinblastine Colchicine	Human cancer cell lines (breast, glioblastoma, lung, ovarian, prostate, etc.)	Inhibition of HIF-1α in vitro at the translational level and downstream microtubule disruption, leading to VEGF down-regulation.	Mabjeesh *et al.* (2003) [97] Escuin *et al.* (2005) [98]

Table adapted from Pasquier E, Honore S, and Braguer D. Microtubule-targeting agents in angiogenesis: where do we stand? Drug Resistance Updates. 2006; 9:74-86. Copyright (2006), with permission from Elsevier.

In summary, bleomycin is a chemotherapeutic drug that has been employed to treat haemangiomas of infancy with promising success. Hemangiomas are benign vascular tumours characterised by excessive angiogenesis. No definitive treatment exists for these tumours, and elucidation of bleomycin's mode of action may contribute to the advancement of research for better treatment options for haemangiomas.

In general, bleomycin exerts its activity primarily by inducing single and double stranded DNA breaks. Mitomycin, another chemotherapeutic drug employed in a variety of cancers, is a DNA alkylating agent which was shown to inhibit endothelial cell proliferation. Cytochalasin D, one of the most well studied actin-disrupting drugs, was also shown to inhibit endothelial cell growth. The effects of these three drugs on angiogenesis have not been well documented.

On the other hand, microtubule-disrupting drugs have been reported to inhibit a wide range of endothelial cell functions associated with angiogenesis, including cell growth, migration and tube formation. Some of these cytoskeletal-disrupting drugs induce apoptosis in a wide range of tumour cell lines. Apoptosis inhibition is an important requirement for angiogenesis, and is exerted by a number of angiogenic growth factors, including VEGF, and bFGF. Some of the potent angiogenesis inhibitors in clinical trials, such as angiostatin, induce endothelial cell apoptosis.

Given the fact that the various drugs inhibit aspects of the angiogenesis process, it was considered imperative that their roles in angiogenesis be investigated to further determine whether they may have potential in the treatment of haemangiomas.

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CHAPTER 3

A rapid High-Performance Liquid Chromatographic (HPLC) method for the detection of bleomycin A_2 and B_2 in human plasma

3.1. Introduction

Bleomycin is a well-known chemotherapeutic drug used in the treatment of squamous cell carcinomas, testicular cancer and malignant lymphomas. [1] It is devoid of myelotoxicity and cardiotoxicity, and does not cause diarrhea, vomiting or nausea. [2] Its anti-neoplastic effect is via oxidant damage to DNA. [3]

The major side effect of bleomycin following intravenous administration is the development of pulmonary fibrosis. [4,5] Alveolar capillary membrane damage due to free radical formation has been cited as one of the factors resulting in the development of pulmonary fibrosis.[6] It is for this very reason that bleomycin is widely used to induce lung injury in various animal models with resultant oxidant-induced inflammation and fibrotic lesions in the lung interstitium.[3] Although bleomycin-induced pulmonary toxicity is generally considered to be a dose related side-effect, it has been documented that pulmonary fibrosis can occur with any bleomycin dosage. [6]

Bleomycin has more recently, been employed to treat haemangiomas of infancy, with very good results. [1,7,8] However, concerns about the use of chemotherapy to treat benign tumours and the possible development of bleomycin induced pulmonary fibrosis in such patients remain. Indeed, the plasma concentration of bleomycin following intralesional injection into vascular lesions is unknown. The monitoring of bleomycin levels in body fluids following intralesional therapy is imperative for the establishment of safety parameters for its use.

Various analytical methods have been developed to assay bleomycin in biological fluids. [2-6,13] Broughton and Strong in 1976 used a radioimmunoassay method to assay this compound in phosphate buffered saline (PBS) and in serum. [5] However, bleomycins are a mixture of active fractions (A_1-A_6; B_1-B_5), and the pharmacology of the different composite fractions are clinically important. [14]

Furthermore, clinically administered bleomycin (bleomycin sulphate USP, or Blenoxane™ in the case of this study) consists of, by weight, 55-70% bleomycin A_2, 25-32% bleomycin B_2, and the remaining percentage divided among the other sub-fractions. [10] This radioimmunoassay method was inadequate as it did not distinguish between the various components of the bleomycin mixture. [13] Back in 1979, Shiu *et al*. motivated for the development of an assay method for all the major components of bleomycin. [2]

In 1980 Shiu and Goehl published a high performance liquid chromatography (HPLC) method for the specific determination of one of the major component of the bleomycin mixture, namely bleomycin A_2, in plasma. [6]

Ten years later another group developed a more sensitive HPLC method using a fluorescence detector in a linear gradient, ion-paired reversed phase procedure to assay bleomycin A_2 in human plasma and rat hepatocytes. [14] These HPLC methods for the determination of bleomycin in plasma were validated for the A_2 fraction only. Furthermore, these long assay methods would not be optimally applicable for monitoring a large number of patients' plasma samples.

In the present study, a rapid high performance liquid chromatographic method for the separation and quantitation of both major fractions, bleomycin A_2 and B_2, in human plasma was developed. This method was then employed to determine levels of bleomycin in patients treated with intralesional bleomycin.

3.2. Materials and Method

3.2.1. Reagents

Bleomycin A_2 (BWS-18) was donated by the National Institute of Health, Japan, and Bleomycin B_2 (BMT 049 B2), was donated by Nippon Kayaku, Co, Ltd. (Japan). Methanol and acetonitrile were of HPLC grade; acetic acid was of reagent grade (Radchem, Johannesburg, SA). Sodium heptanesulfonate was purchased from African Biotech Consultants (Johannesburg, SA). Water was purified by a MilliQ water purification system.

3.2.2. Apparatus

A Waters™ LC Module1High Performance Chromatograph (HPLC) was used. The HPLC system and conditions are summarized in Table 3.1.

3.2.3. Preparation of mobile phase

The mobile phase consisted of water-0.0085 M sodium heptanesulfonate: acetonitrile: acetic acid (70:25:5). MilliQ purified water used to prepare the mobile phase was 18 mega ohm quality. The final pH of the mobile phase solution was 4.7. The solution was filtered through a 0.45 nylon membrane to remove contaminants. The solution was then degassed prior to transfer to an HPLC solvent bottle. Throughout the analysis, the mobile phase was sparged with helium at a rate of 500 ml/min to prevent the formation of air-bubbles, which can cause a considerable drop in backpressure.

For the assay of bleomycin fractions, a mobile phase flow rate of 1 ml/min was established. The resulting backpressure was approximately 2000 psi. The temperature of the column was maintained at 40^{o}C during separation.

3.2.4. Bleomycin Stock

Bleomycin stock solutions were prepared at 0.8 mg/ml bleomycin A_2 and 1 mg/ml bleomycin B_2 in 0.1 M sodium-phosphate buffer, pH 6.8. The stock solutions were kept at -20^{O}C until use. Standard solutions of bleomycin fractions were prepared from stock solutions to calibrate the system prior to the assay of samples.

Table 3.1. Summary of the HPLC system and analytical conditions.

Hardware and Analytical Conditions	**Specifics**
Auto sampler	LC Module-1(Waters Corp., Milford, USA)
Pump	Single piston; constant flow W 600
Injector	WISP model 715; Valve-type, electric driven backfill injector (Waters, USA)
Detector	UV-Vis W486 signal detector; light source = deuterium discharge lamp covering 200-350 nm UV range (Waters, USA)
Guard column	Phenomenex C18; 4mm L x 2 mm ID (AJO – 4286); zero dead volume (Waters, SA)
Column	LUNA™ C18(2) 250 mm L x 4.6 mm ID (Phenomenex Corp., Sunnyvale, CA, USA)
Interphase box	Hercules Lite (Varian Inc., Palo Alto, CA, USA)
Software	Galaxie 1.5 (Varian Inc., Palo Alto, CA, USA)
Room temperature	25 ºC
HPLC pressure	1900 - 2100 p.s.i.
Sample volume injected	5 µl for standards; 20µl for samples
Flow rate	1 ml/min
Mobile phase	water-NaHS:acetonitrile: acetic acid; 70:25:5

NAHS – sodium heptane sulphonate

3.2.5. Preparation of standards

Frozen stock solutions were thawed and diluted with milli-Q water to obtain a range of concentrations (1, 2, 4, 6, 8, 200, 250, 300 µg/ml bleomycin A_2; 2, 4, 6, 8, 10, 300, 350, 400 µg/ml bleomycin B_2) required for the preparation of plasma standards. Standards were prepared by adding 10 µl of bleomycin A_2 or B_2 to 100 µl of drug free plasma. Hence, the corresponding plasma standards were 0.1, 0.2, 0.4, 0.6, 0.8, 20, 25, 30 µg/ml bleomycin A_2; 0.2, 0.4, 0.6, 0.8, 1, 30, 35, 40 µg/ml bleomycin B_2. Levels above 160 µg/ml bleomycin were measured in the patient samples. Therefore another standard curve was created at concentrations of 100, 200, 400, 600, 800 µg/ml A_2; 100, 200, 400, 600, 800, 1000 µg/ml B_2. The system was calibrated daily with the standards prior to analysis to account for interday variations in experimental conditions.

3.2.6. Assay procedure

The samples were vortexed for 30 seconds and then centrifuged for 10 minutes at 3000 rpm. The supernatant was filtered twice through a 0.2 µm cellulose acetate filter. For each dilution, 20 µl of the filtered plasma sample was injected on to the column.

3.2.7. Validation of the assay method

Validation is the presentation of documented evidence that all causes for variation have been accounted for, and that any variation present will not be excessive of expected variation or standard curve variation. [14] The main analytical variables for the validation of a HPLC method are accuracy, linearity, specificity, precision and sensitivity.[15]

3.2.7.1. Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between a value which is accepted either as a conventional true value or an accepted true value and the measured value. [14] In this study, the accuracy of the method was determined by comparing the peak heights resulting from spiked plasma standards, and the peak heights obtained from direct injection of the same amount of drug in aqueous solution.

3.2.7.2. Linearity

The linearity of an analytical procedure is the ability (within a given range) to obtain test results that are directly proportional to the concentration of analyte in the sample. [14] Linearity was established for 5 concentrations across the range of the analytical procedure. The linearity of this assay was assessed by comparison of calibration curves from analyses of spiked samples of bleomycin A_2 at 0.1 to 0.8 µg/ml and of bleomycin B_2 at 0.2 to 1.0 µg/ml on three different days.

3.2.7.3. Specificity

Specificity is the ability to unequivocally assess the analyte in the presence of components that are expected to be present. Identification tests were performed by injecting each entity separately into the HPLC.

3.2.7.4. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. [14]

Aliquots of plasma were spiked with known amounts of the bleomycin stock solution to give a range of concentrations (table 3.3). The plasma samples were assayed in triplicate. The mean, standard deviation, the coefficient of variation (CV) and relative error for the assay were calculated.

3.2.7.5. Limit of detection (LOD)

The Detection limit is the lowest amount of analyte in a sample which can be detected, but not necessarily quantitated as an exact value. [14] The LOD was based on the standard deviation of the response curve and the slope, and was expressed as follows:

$$LOD = \frac{3.3\sigma}{S}$$

where, σ = the standard deviation of the response

S = the slope of the calibration curve

3.2.7.6. Limit of quantitation (LOQ)

The quantitation limit is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. [14] The LOQ was based on the standard deviation of the response curve and the slope, and was calculated as follows:

$$LOQ = \frac{10\sigma}{S}$$

where, σ = the standard deviation of the response

S = the slope of the calibration curve

3.2.8. Patient s

In this study which was approved by the University of Pretoria ethics committee (IPA19-96/2000), blood samples were obtained from ten patients with vascular anomalies treated with bleomycin. Informed consent was obtained from all patients or guardians (in the case of children younger than 18 years). Intralesional bleomycin was used for the treatment of four patients with haemangiomas at dosages of 0.2-0.94 mg/kg/therapy. Patient information is tabulated in table 3.4.

The bleomycin sulphate formulation used in this study was Blenoxane (Bristol Myers Squibb, Bedfordview, South Africa), which contains approximately 69% bleomycin A_2 and 29% bleomycin B_2.[16] Intralesional injection was followed by the local application of pressure for 10 minutes, after which, where possible, a pressure dressing was applied.

The diagnosis of haemangioma was based on the medical history and physical examination of the patients. Magnetic Resonance Imaging provided further useful information on the location, extent, and involvement of deeper structures in more complex haemangiomas. Blood samples were taken from the patients before IB and thereafter at 10 and 30 minute intervals, followed by further sampling at 1, 6, 24 hours after intralesional therapy. As a control, blood samples were also taken from four cancer patients who received bleomycin intravenously at doses of 0.2-0.5 mg/kg/therapy, and at the same time intervals as for the haemangioma patients.

3.2.9. Patient Sample Analysis

Blood samples were centrifuged at 2500 rpm for 5 minutes. Aliquots of plasma were transferred to 5ml tubes. The plasma samples were deproteinised with methanol, 1 part in 2, and centrifuged at 3000 rpm for 10 minutes. The supernatant was then filtered and dispensed into 3 ml vials. Samples were analysed using HPLC. All patient samples were prepared and assayed in duplicate.

3.3. Results

3.3.1 Method development

Bleomycin levels were determined in a stock solution of the drug prepared in aqueous solution. Peaks of bleomycin A_2 and B_2 were separated successfully. Using assay conditions described under Materials and Methods, BLM A_2 and BLM B_2 had retention times of approximately 5.22 and 5.86 minutes respectively. No bleomycin was detected in control human plasma, (plasma from a person who has never been treated with bleomycin). Bleomycin was also measured in human plasma spiked with varying concentrations of the drug. Chromatograms are represented in Fig 3.1.The accuracy and precision for the assay are reported in tables 3.2 and 3.3.

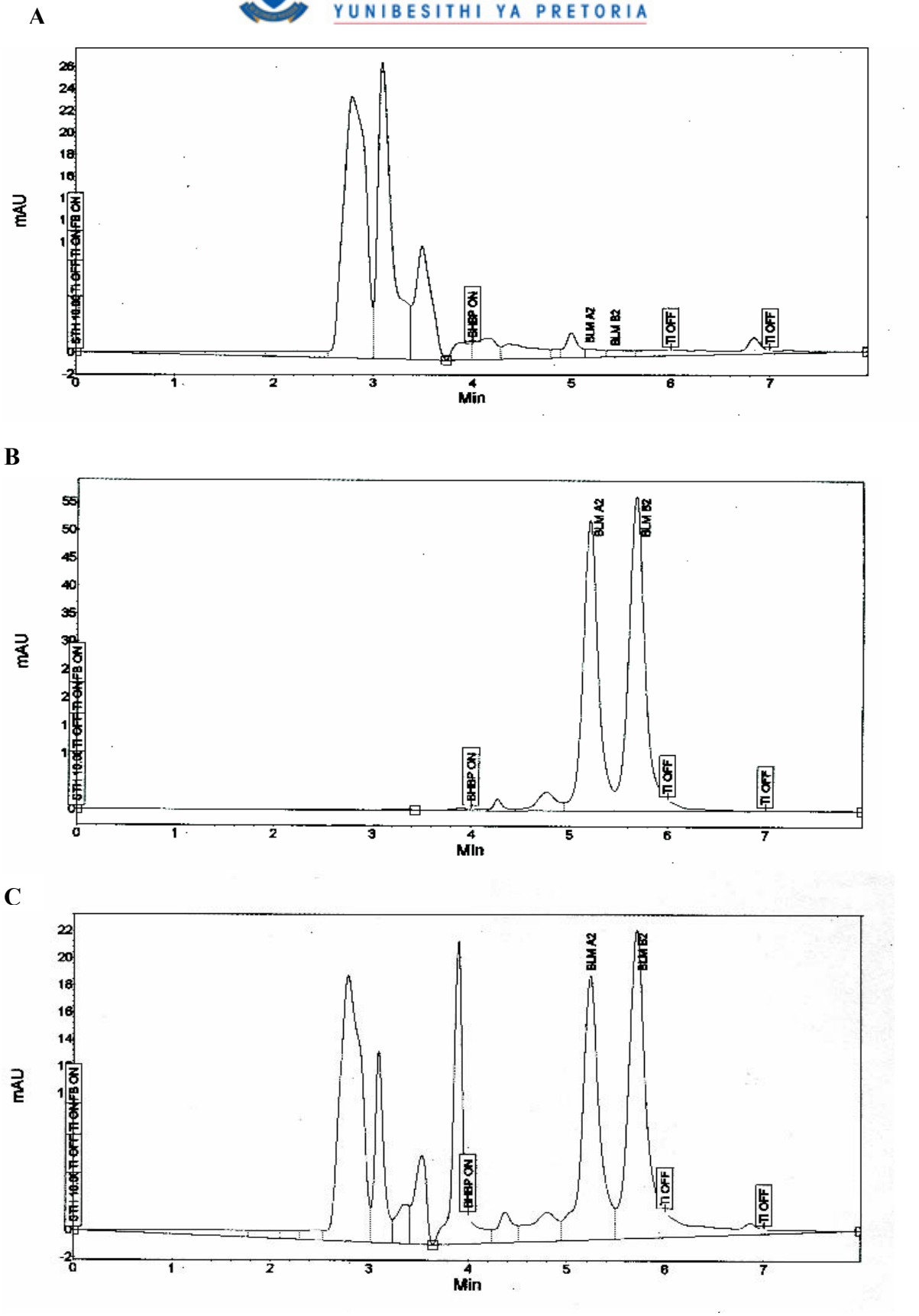
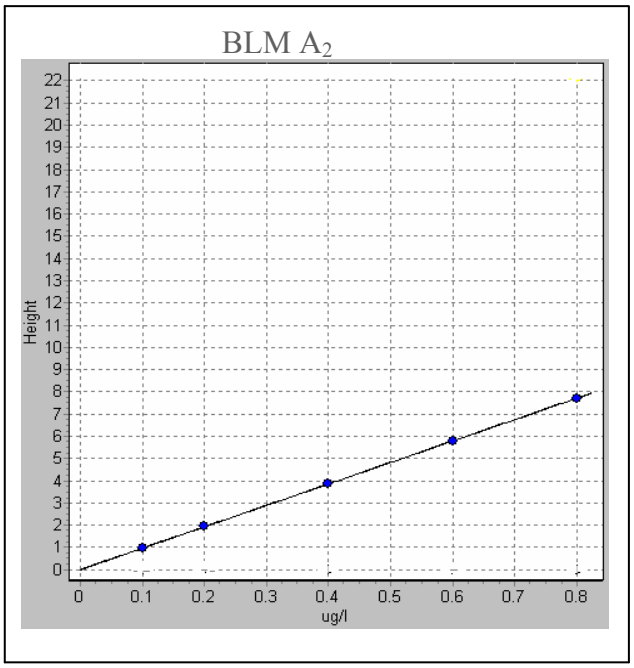
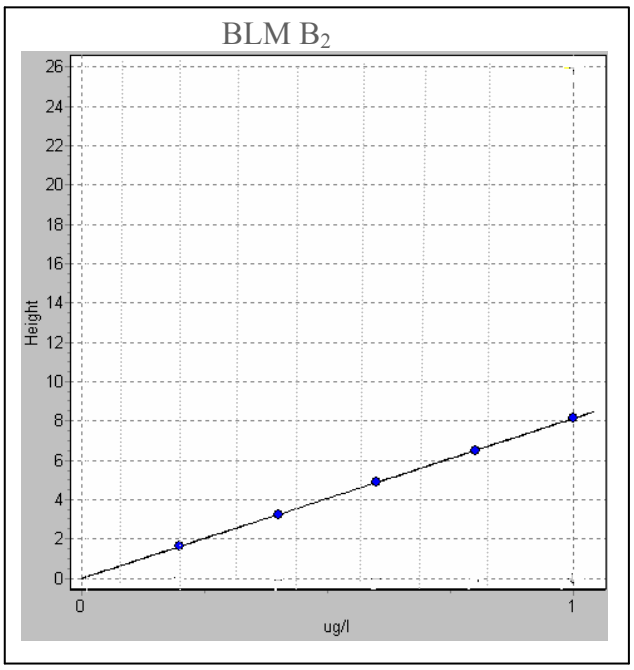


Figure 3.1. Chromatograms (absorbance in mAU versus time in minutes) obtained from the analysis of (A) Control human plasma, (B) Bleomycin stock in aqueous solution, and (C) Human plasma spiked with bleomycin A_2 (20 µg/ml) and bleomycin B_2 (30 µg/ml).



Component : BLM A_2
Polynom : y = bx + a
a = 0
b = 9.63802
Correlation coef. : 1.0000

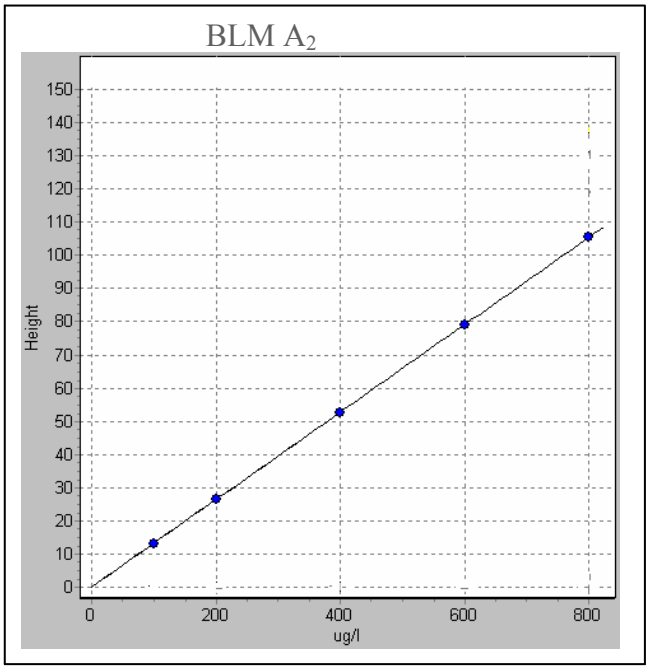
Y : Peak height (response)
X : BLM concentration



Component : BLM B_2
Polynom : y = bx + a
a = 0
b = 8.13273
Correlation coef. : 1.0000

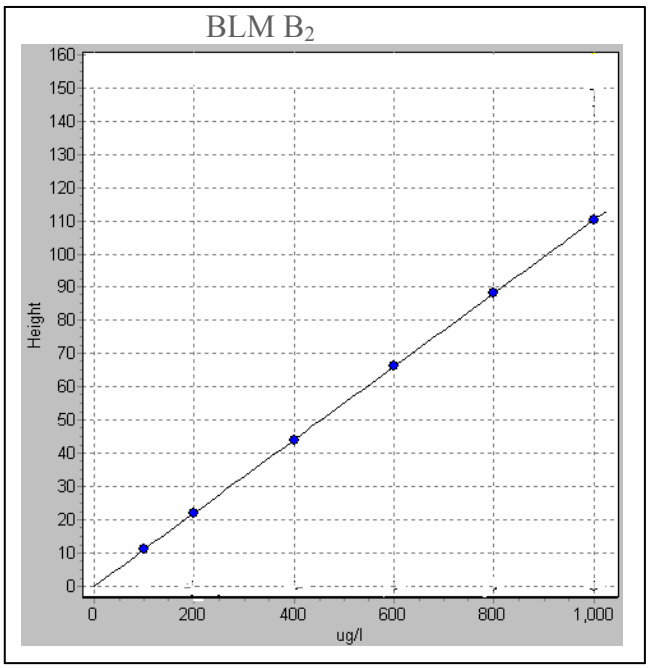
Y : Peak height (response)
X : BLM concentration (µg/ml)

Figure 3.2. Calibration curves for bleomycin A_2 and B_2 (0.1-2.0 µg/ml). The curves were plotted following the injection of plasma standards in the concentration range of 0.1-0.8 and 0.2-1 µg/ml bleomycin A_2 and B_2 respectively.



Component : BLM A_2
Polynom : y = bx + a
a = 0
b = 0.13183
Correlation coef. : 1.0000

Y : Peak height (response)
X : BLM concentration (µg/ml)



Component : BLM B_2
Polynom : y = bx + a
a = 0
b = 0.11018
Correlation coef. : 1.0000

Y : Peak height (response)
X : BLM concentration (µg/ml)

Figure 3.3. Calibration curves for bleomycin A_2 and B_2 (100-1000 µg/ml). The curves were plotted following the injection of plasma standards in the concentration range of 100-800 and 100-1000 µg/ml bleomycin A_2 and B_2 respectively.

Table 3.2. Accuracy of the bleomycin HPLC assay method.

FRACTION	ACTUAL CONCENTRATION (µg/ml)	EXPERIMENTAL CONCENTRATION (µg/ml)	RELATIVE RECOVERY (%)
BLM A_2	30	31.1 ± 0.16	103.7
BLM B_2	40	39.0 ± 0.65	97.5
BLM A_2	25	26.8 ± 0.51	107.2
BLM B_2	35	34.5 ± 0.30	98.7
BLM A_2	20	25.1 ± 0.51	125.5
BLM B_2	30	31.8 ± 0.21	106.0

n = 3; the actual concentration represents the amount of BLM A_2/ B_2 injected onto the column, and the experimental concentration is the amount of BLM A_2/ B_2 measured.

To study the precision of the method, known amounts of the bleomycin stock were added to control plasma. The samples were assayed in triplicate. The intra-assay coefficient of variation (CV) ranged from 0.52 to 2.03 for BLM A_2, and from 0.66 to 2.08 for BLM B_2. The mean relative error was 1.50 for BLM A_2 and 2.17 for BLM B_2. The limit of detection was 33 ng/ml for BLM A_2 and 65 ng/ml for BLM B_2

Table 3.3. Precision of the assay method for BLM A_2 and BLM B_2 in plasma.

FRACTION	MEAN CONCENTRATION	CV	% ERROR
BLM A_2	30	0.52	0.64
BLM B_2	40	1.67	3.77
BLM A_2	25	1.90	1.86
BLM B_2	35	2.08	0.66
BLM A_2	20	2.03	1.99
BLM B_2	30	0.66	2.08

(n=3)

3.3.2. Levels of bleomycin in patient samples

Plasma levels of bleomycin fractions (bleomycin A_2 and B_2) measured in all patients after administration are listed in Table 3.4. None of the fractions were detected in the samples collected over a 24 hour period after intralesional bleomycin treatment of haemangioma patients. Relatively high levels of bleomycin fractions were measured in the plasma of cancer patients at 10 min, 30 min, 1 hr, 6 hr and 24 hr following IV therapy. The overall mean levels of bleomycin A_2 and B_2 over the full 24-hour period were 360.79 and 183.57 µg/ml in the cancer patients.

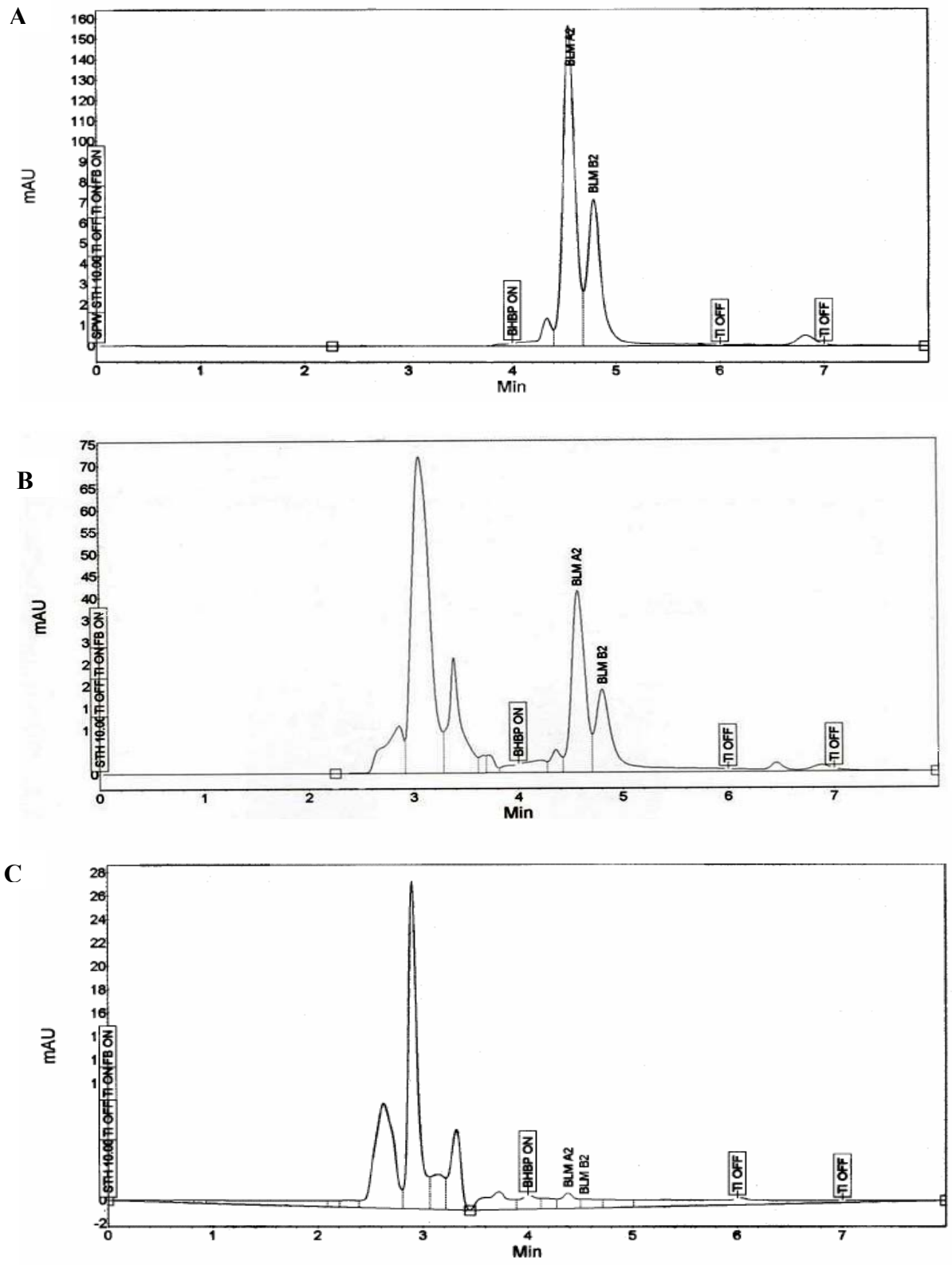


Figure 3.3. Chromatograms (absorbance in mAU versus time in minutes) obtained from the analysis of: (A) Blenoxane (clinically formulated bleomycin) reconstituted at 1mg/ml in sterile 0.9% saline, (B) a blood sample obtained from a lymphoma patient 1 hour after IV bleomycin treatment; (C) a blood sample obtained from a haemangioma patient 1 hour after IB treatment.

Table 3.4. Mean levels of bleomycin fractions, bleomycin A_2 and B_2, in plasma samples obtained over a 24hr period from haemangioma and cancer patients. Results are mean ± SEM; n = 2

PATIENT	**AGE**	**DIAGNOSIS**	**DOSE (mg/kg)**	**BLM A_2 (µg/ml)**	**BLM B_2 (µg/ml)**
1	10 M	Haemangioma	0.94 IB	0.00	0.00
2	6 M	Haemangioma	0.60 IB	0.00	0.00
3	13 M	Haemangioma	0.60 IB	0.00	0.00
4	10 M	Haemangioma	0.20 IB	0.00	0.00
5	10 Y	Hodgkin's Lymphoma	0.40 IV	273.16 ± 121.26	164.44 ± 63.86
6	12 Y	Hodgkin's Lymphoma	0.50 IV	678.44 ± 164.7	237.48 ± 83.92
7	14 Y	Hodgkin's Lymphoma	0.20 IV	211.95 ± 106.56	99.98 ± 51.24
8	15 M	Kaposis Sarcoma	0.50 IV	279.60 ± 37.63	129.53 ± 22.02

3.4. Discussion

Systemic toxicity after intralesional bleomycin therapy in haemangioma patients has not been previously reported. Nevertheless, the potential for bleomycin-induced pulmonary toxicity remains a major concern in such patients. As an initial study into these potential effects, the determination of the blood level spill-over and peak values after intralesional bleomycin injection of these vascular lesions was considered imperative.

A high-performance liquid chromatographic method for the analysis of bleomycin A_2 and B_2, the two major fractions constituting Blenoxane, was therefore developed. The method was based on the ability to force drugs into their ionic forms by pH adjustment and then additing of counter-ions to form ion-pair complexes.

The mobile phase consisted of water-sodium heptanesulphonate (NaHS):acetonitrile: acetic acid mixed together in the ratio 70:25:5. Acetic acid was the component added to force the drug to be separated into its ionic form, and also to preserve the stationery phase, because above pH 7 the silica (which constitutes the stationery phase) disintegrates. [11]

Bleomycins are highly hydrophilic, and thus it is difficult to avoid interference from polar endogenous substances in plasma during separation on a reversed phase HPLC system. Sodium heptanesulfonate was thus used as an ion-pairing reagent to separate bleomycin from nearly equally hydrophilic endogenous compounds in plasma. [11]

Prior to sample analysis using the HPLC, a one-step protein precipitation sample preparation procedure was used to remove proteins from the plasma. Because of the simple nature of this sample preparation method, no internal standard was used. Deproteinised samples were then filtered through a 0.2 µm cellulose acetate filter to remove any interfering substances. This filter was used because in previous studies, the filtration of bleomycins through a cellulose ester membrane indicated insignificant adsorption, and no reduction in the potency of the drug was observed. Following the filtration step, the samples were injected onto the HPLC column.

Discrete separation of two bleomycin analogues, A_2 and B_2, from human plasma was achieved using isocratic gradient chromatographic conditions described in the methods section. No peaks representing interfering plasma components were observed in chromatograms of unspiked plasma (Fig 3.1A), indicating that the method was specific for bleomycin.

Using the above assay conditions, elution times were reduced considerably; BLM A_2 and BLM B_2 had retention times of 5.22 and 5.86 minutes respectively. Previously reported work on the HPLC separation of bleomycin A_2 required long elution times of up to 45 minutes.

With the current method, it took a total HPLC run-time of 8 minutes for both elution of the sample and equilibration of the system. In addition, the resolution between BLM A_2 and BLM B_2 was good (R = 1.8). The relative recovery of bleomycin A_2 and B_2 was approximately 100%. The limit of detection was 33.3 ng/ml for BLM A_2, and 65 ng/ml for BLM B_2. The limit of quantitation was 0.1 and 0.2 µg/ml for bleomycin A_2 and B_2 respectively. A good linear relationship between peak height and concentration ($r^2 = 1$) was obtained for BLM A_2 and BLM B_2 over the concentration ranges of 0.1 to 0.8 µg/ml and 0.2 to 1 µg/ml (fig 3.2) respectively. A good linear relationship was also obtained with over the concentration range of 800 to 1000 µg/ml (fig 3.3). The reason for including the latter concentration range was because in preliminary studies on the assay of bleomycin samples obtained from a cancer patient following intravenous injection, bleomycin levels in this range were measured.

The assay method developed in this study was subsequently used to determine bleomycin levels in plasma samples of patients receiving the drug either intralesionally or intravenously. Chromatograms obtained from the analysis of patient plasma are represented in fig 3.4.

In this study, the total amount of bleomycin injected in patients treated with bleomycin intralesionally was between 0.2 - 0.9 mg/kg, which is in the same range as doses used in cancer therapy (0.2 - 0.5 mg/kg). However, injection was intralesional for haemangiomas whereas it was systemic (intravenous) in the cancer patients.

The mean levels of bleomycin A_2 and B_2 measured in samples of IB-treated patients over the full 24-hour period were 0 µg/ml for both fractions and 360.79 and 183.57 µg/ml bleomycin A_2 and B_2 respectively in samples of cancer patients treated with intravenous bleomycin (Table 3.4).

From these results it was apparent that the plasma bleomycin concentrations in this study of haemangioma patients receiving bleomycin *intralesionally* were more than 100 times lower than plasma bleomycin concentrations obtained from samples of the cancer patients receiving bleomycin *intravenously*. Findings indicate that the low levels detected may translate to a significantly lesser risk of pulmonary fibrosis following IB therapy. These observations may be indicative of the safety of the drug regime.

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CHAPTER 4

The effects of bleomycin, mitomycin C, and multiple cytoskeletal-disrupting agents on endothelial cell migration and growth

4.1. Introduction

Angiogenesis, the formation of new capillary blood vessels by a process of sprouting from pre-existing vessels, is required for the maintenance of functional and structural integrity of the organism. [1,2] In the healthy adult organism, the endothelial cell turnover is very low, and occurs during the female reproductive cycle (in the corpus luteum and endometrium), in the placenta and mammary glands during pregnancy, during the wound healing process, and in response to tissue hypoxia. [3,4,5] Angiogenesis in these situations is tightly regulated.

Angiogenesis also occurs in pathological situations, such as juvenile haemangiomas, is necessary for the continued growth of tumours, and contributes to the hematogenous spread of tumour cells and the formation of metastasis. [2,5] Thus the identification of agents which inhibit angiogenesis, and the elucidation of the mechanism of action of such agents, represents an important therapeutic objective.

The process of angiogenesis begins with localized breakdown of the basement membrane of the parent vessel (usually a postcapillary venule). [5,6] Endothelial cells then migrate into the surrounding matrix within which they form a capillary sprout. The sprout elongates by further migration and by endothelial cell proliferation proximal to the migrating front, and a lumen is gradually formed proximal to the region of proliferation. [5-7]

Angiogenesis is thus characterized by alterations in at least three endothelial cell functions, each of which is a potential target for antiangiogenic strategies: (1) modulation of interactions with the extracellular matrix, (2) an initial increase and subsequent decrease in migration, and (3) an increase in proliferation, which provides new cells for the growing and elongating vessel. [5, 7]

In addition, structural interactions between the extracellular matrix and the different cytoskeletal filaments (microfilaments and microtubules) appear to play an important role in cell growth during angiogenesis. [8]

The purpose of the present chapter was to investigate the effects of DNA-damaging drugs (bleomycin and mitomycin C) and cytoskeletal-disrupting agents (2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole and cytochalasin D) on two of the endothelial cell functions that are important in angiogenesis, namely, endothelial cell migration and endothelial cell growth.

4.2. Methods

4.2.1. Cell culture maintenance

Experiments were conducted on primary bovine adrenal cortex-derived microvascular endothelial (BME) cells, and human dermal microvascular endothelial cells (HMEC-1), which were a generous gift from the Centre for Disease Control, USA. The cell lines were maintained as monolayer cultures in a 37 ^{o}C incubator in a humidified atmosphere containing 5% CO_2. BME cells were grown in α -modified minimum essential medium (α-MEM) supplemented with 15% Donor Calf Serum (DCS), 500 U/ml penicillin and 100 µg/ml streptomycin; [9] HMEC-1 were maintained in MCDB-131 medium (Laboratory Specialist Services, Cape Town, SA), supplemented with 1% penicillin-streptomycin (Highveld Biologicals, Sandringham, SA), 10% Fetal Bovine Serum (Highveld Biologicals, Sandringham, SA), 2 mM L-Glutamine (Sigma Chemical CO, St Louis, MO, USA), 1 µg/ml hydrocortisone, and 10 ng/ml Epidermal Growth Factor (Sigma Chemical Co., St Louis, MO, USA).

Viable HMEC-1 from stock flasks were determined using trypan blue exclusion prior to seeding procedures: cells from stock flasks were trypsinised and the cell suspension stained 1:1 with 0.2% trypan blue in phosphate buffered saline (PBS), both supplied by Sigma Chemical Co., St Louis, MO, USA. Unstained and thus viable cells were counted with a haemocytometer.

4.2.2. Endothelial Cell Migration

Confluent monolayers of BME cells in 35 mm culture dishes were "wounded" with a blade in such a way as to mark the initial wound edge. Wounded cultures were washed twice with serum-free α-MEM, and serum-free α-MEM/0.1% gelatin and test drugs (bleomycin, mitomycin C, 2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole and cytochalasin D) were added at the indicated concentrations (shown in the results section). After 15 hours, monolayers were stained with 0.1% crystal violet in 20% methanol for 30 min. Fields measuring 1.0 x 1.4mm were photographed under phase-contrast using a Nikon Diaphot TMD inverted photomicroscope. The total number of cells which had crossed the original wound edge was determined. Successive parallel 100 µm-deep fields were delineated from the original normal edge. Results represent the mean ± SEM.

4.2.3. Endothelial Cell Growth

Cell growth studies were performed to assess the effects of bleomycin, mitomycin C, 2-methoxyestradiol (2-ME), taxol, vincristine, vinblastine, colchicine and nocodazole on endothelial cell growth using crystal violet nuclear staining.

Human dermal microvascular endothelial cells (HMEC-1) were seeded into 24-well culture plates at a density of 10 000 cells per well and exposed to varying concentrations of test drugs. Control cells were exposed to drug vehicles. Growth was terminated after 48 hours by fixation with 1% glutaraldehyde in PBS for 15 minutes. Cells were then stained with a 1% solution of crystal violet (in H_2O) for 30 minutes. The chromophore was extracted with 500 µl of 0.1% Triton X-100 per well. The absorbance of samples was read at 570 nm on an ELx 800 Universal Microplate Reader (Bio-Tek instruments Inc, Weltevreden, SA). Three wells were analysed for each concentration. Growth studies were repeated three times for each drug.

Further cell growth studies were conducted on BME cells using a FACScan Analyzer as previously described. [10] The cells were seeded into gelatin-coated 24 well-plates at a density of 10 000 cells/ well. Cells were grown in α-MEM supplemented with 5% DCS, 24 hours later, fresh medium was added together with test drugs (bleomycin, mitomycin C, 2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole and cytochalasin D). Control cells were exposed to drug vehicles. Two days later, medium and test drugs were renewed, and after a further 2 days, cells were trypsinised and counted in a FACScan Analyzer (Becton-Dickinson, San José, CA).

4.3. Statistical Analysis

Statistics were performed using Stata Release 8. The concentration-dependent effect of the different drugs on endothelial cell cultures was analyzed by comparing control and treated cells. One-way ANOVA was used, and where significance was reached, Bonferroni's method was used. Significance was designated at $P < 0.05$.

4.4. Results

4.4.1. Endothelial Cell migration

Studies of the effects of bleomycin and other test drugs on endothelial cell migration were conducted using BME cells. The effects of test drugs on endothelial cell migration were assessed in a 2-dimensional assay in which migration was measured as the number of cells that have moved into an artificially created wound in a confluent monolayer. Bleomycin inhibited BME cell migration at a dose of 100 µg/ml (fig 4.1). No significant inhibition of endothelial cell migration occurred following exposure of cultures to mitomycin C (fig 4.1).

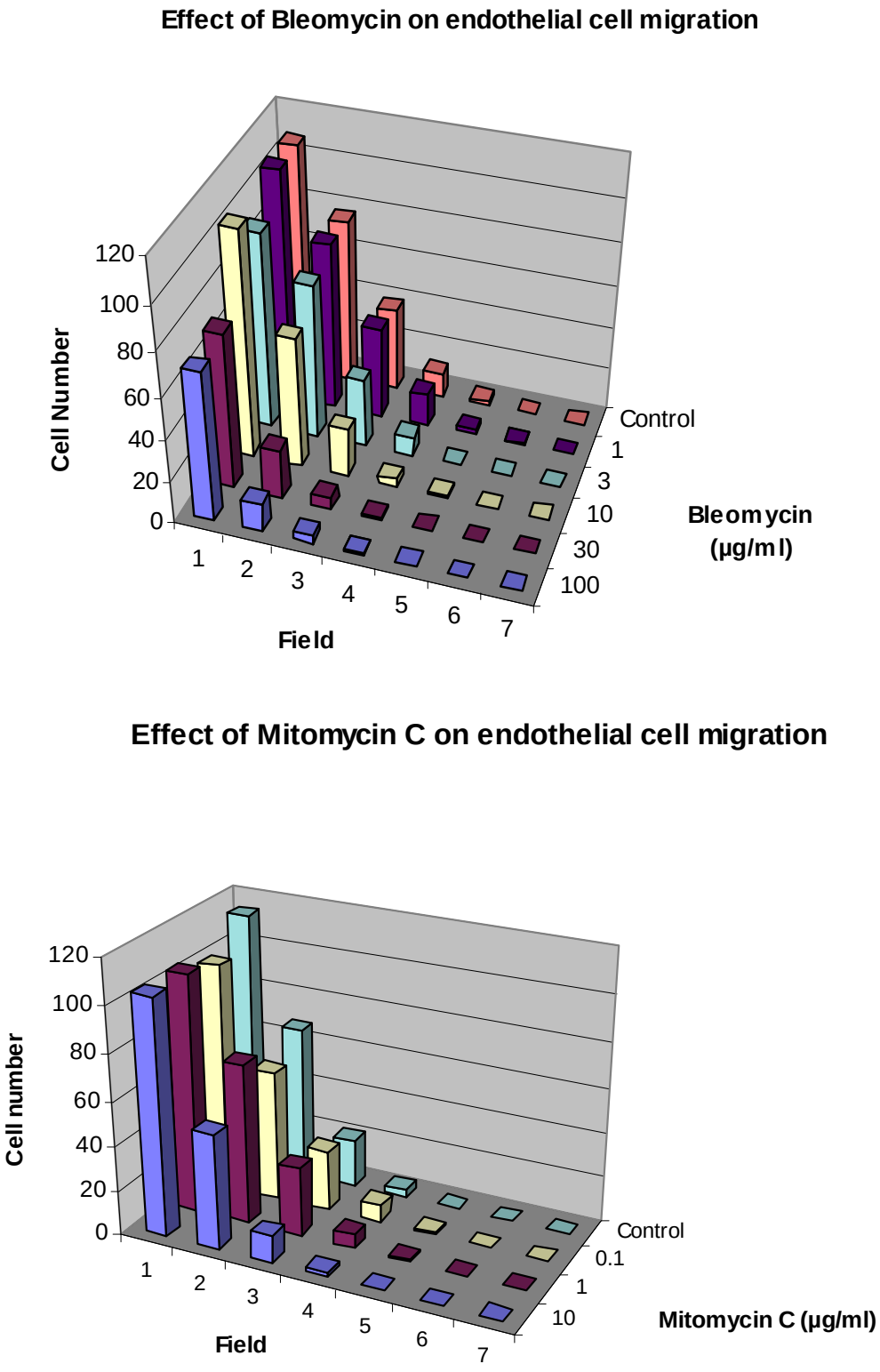


Figure 4.1. Effects of bleomycin and mitomycin C on endothelial cell migration.
Confluent BME cell monolayers were wounded with a blade so as to mark an initial wound edge and washed. Cells were then treated with saline (control), bleomycin or mitomycin C. After 15 hours, cultures were fixed and stained, and subsequently viewed with a light microscope. Within the wound, seven fields measuring 1.0 x 1.4mm were selected. The total number of cells in each field was determined.

Inhibition of endothelial cell migration following exposure to 2-ME occurred at the highest dose (10 µM). Taxol inhibited BME cell migration in a dose-dependent manner, with significant inhibition occurring at 100 nM and maximum inhibition occurring at 1 µM.

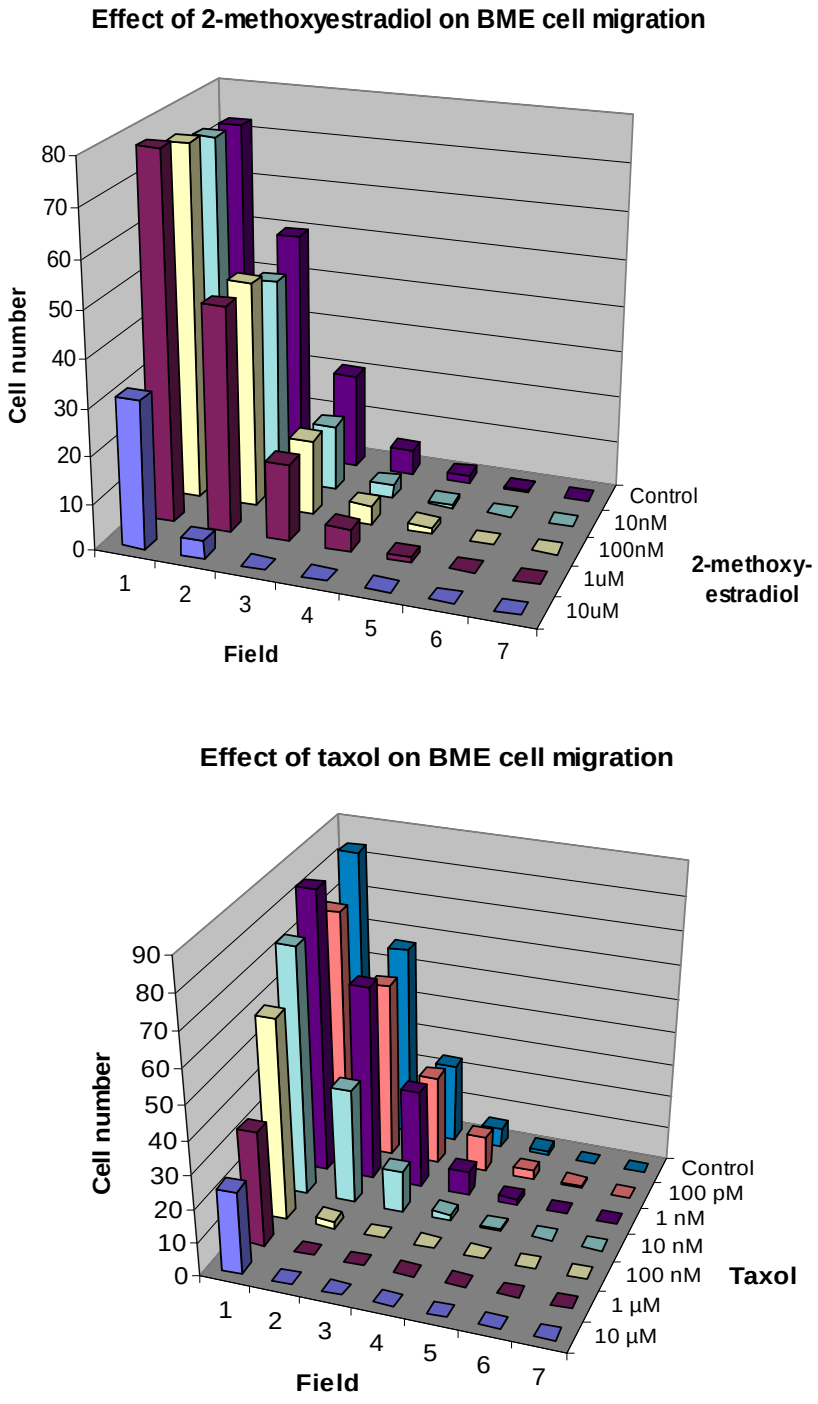


Figure 4.2. Effects of 2-methoxyestradiol and taxol on BME cell migration.
Confluent BME cell monolayers were wounded with a blade so as to mark an initial wound edge and washed. Cells were then treated with DMSO (control), 2-ME or taxol. After 15 hours, cultures were fixed and stained, and subsequently viewed with a light microscope. Within the wound, seven fields measuring 1.0 x 1.4mm were selected. The total number of cells in each field was determined.

Vincristine inhibited cell migration at 1ng/ml, while vinblastine inhibited migration at 10 ng/ml.

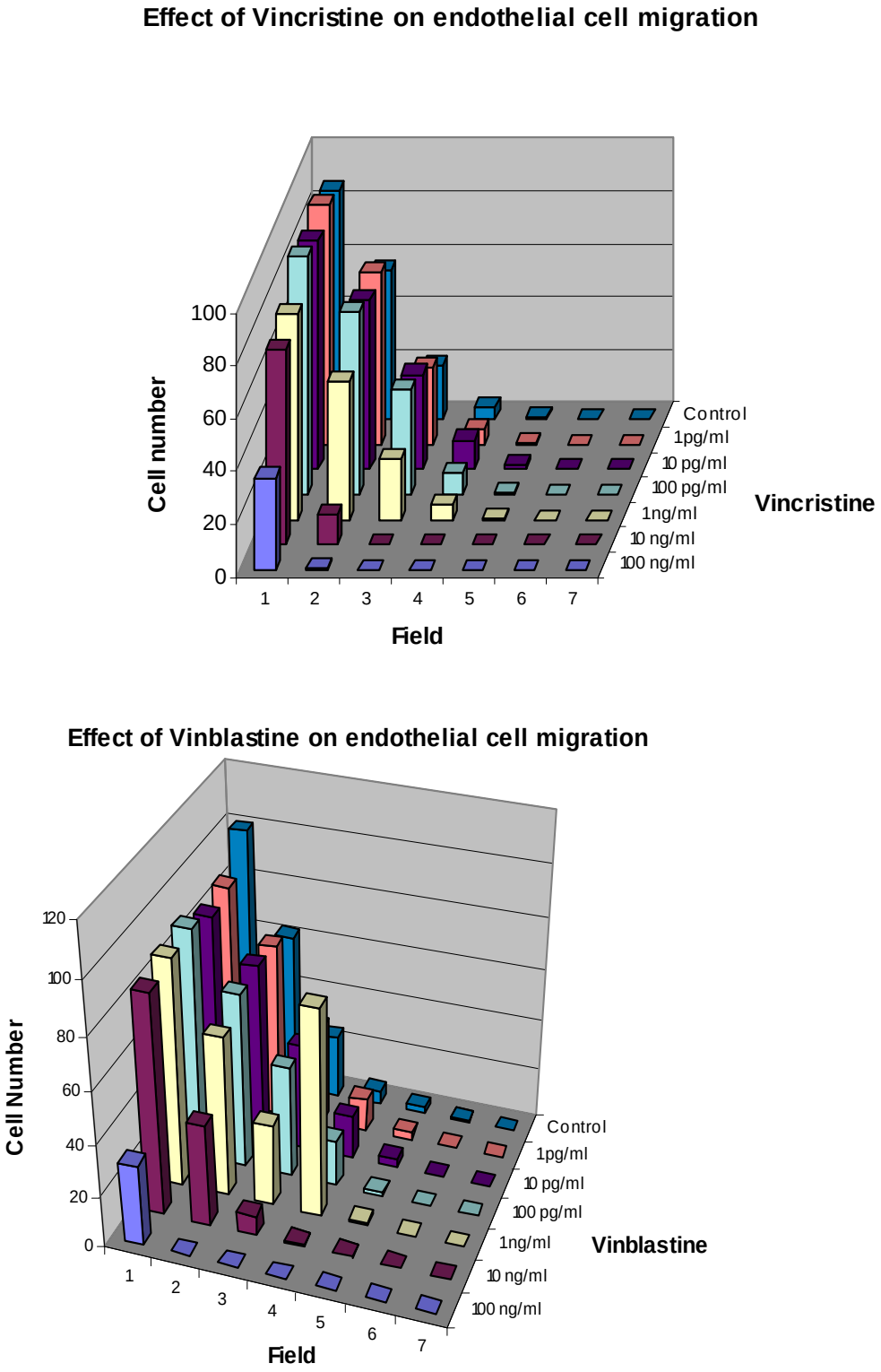


Figure 4.3. Effects of vincristine and vinblastine on endothelial cell migration.
Confluent BME cell monolayers were wounded with a blade so as to mark an initial wound edge and washed. Cells were then treated with saline (control), vincristine or vinblastine. After 15 hours, cultures were fixed and stained, and subsequently viewed with a light microscope. Within the wound, seven fields measuring 1.0 x 1.4mm were selected. The total number of cells in each field was determined.

Colchicine induced a dose-dependent inhibition in cell migration, with significant inhibition occurring at 100 nM. Nocodazole inhibited migration at doses of 10 µM and above.

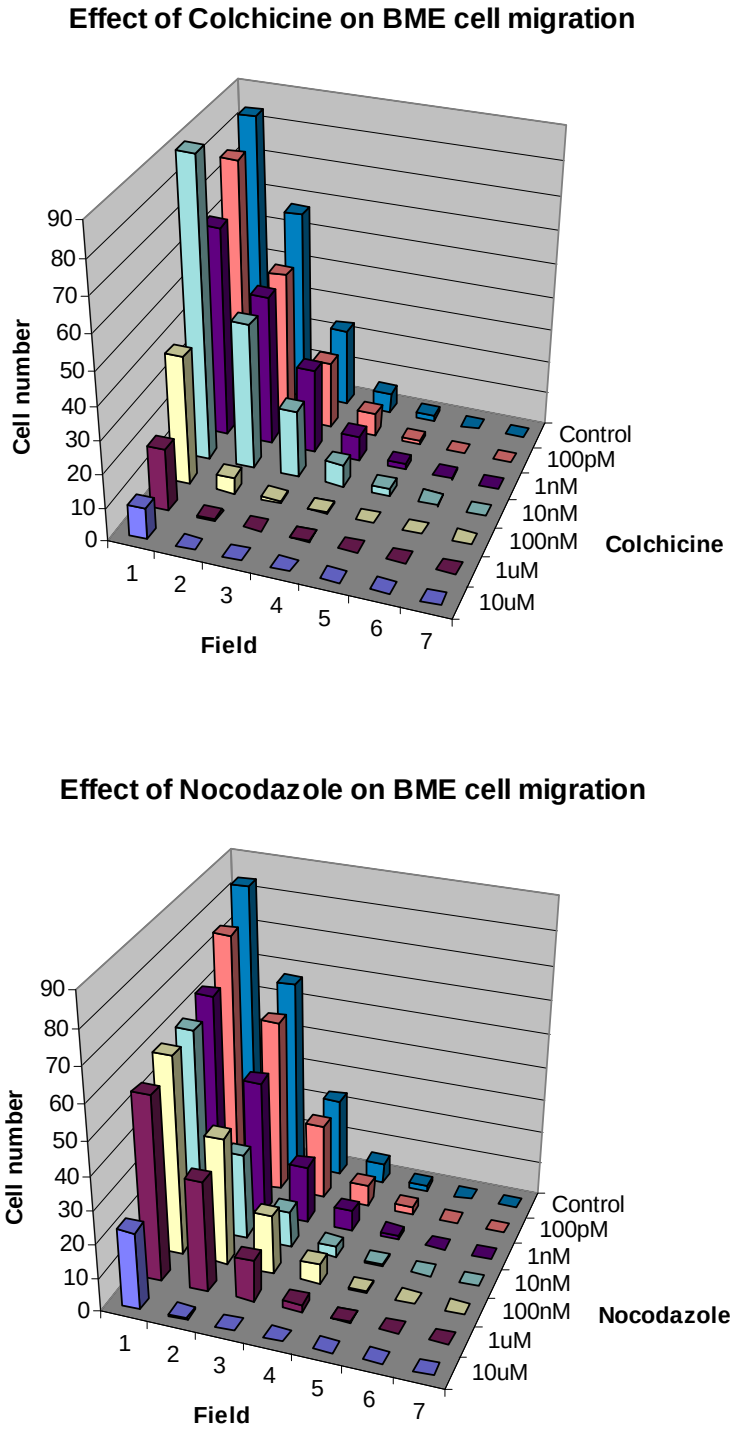


Figure 4.4. Effects of colchicine and nocodazole on BME cell migration.
Confluent BME cell monolayers were wounded with a blade so as to mark an initial wound edge and washed. Cells were then treated with DMSO (control), colchicine or nocodazole. After 15 hours, cultures were fixed and stained, and subsequently viewed with a light microscope. Within the wound, seven fields measuring 1.0 x 1.4mm were selected. The total number of cells in each field was determined.

Cytochalasin D induced a dose-dependent inhibition in cell migration, with significant inhibition occurring at 100 nM.

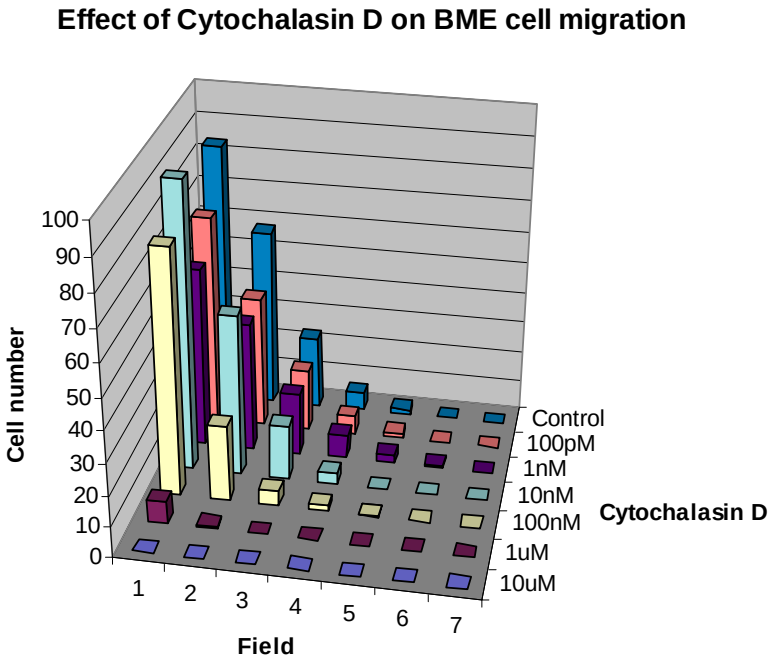
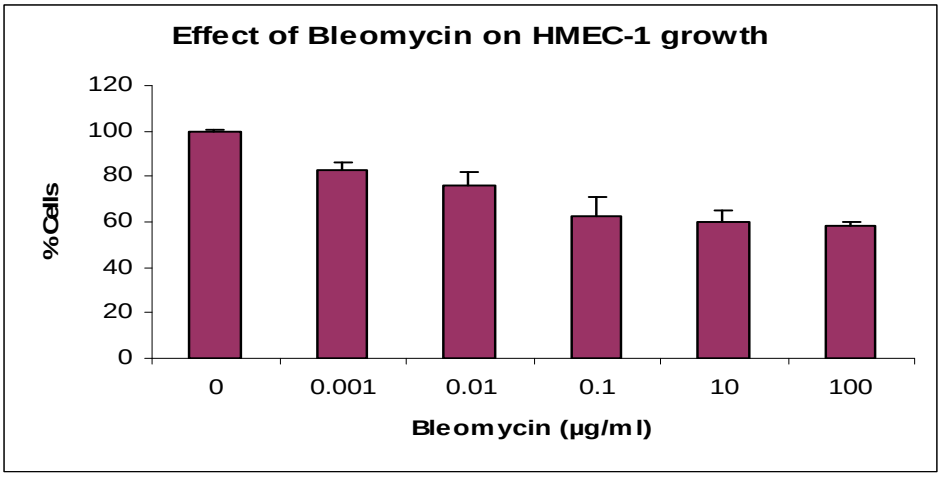
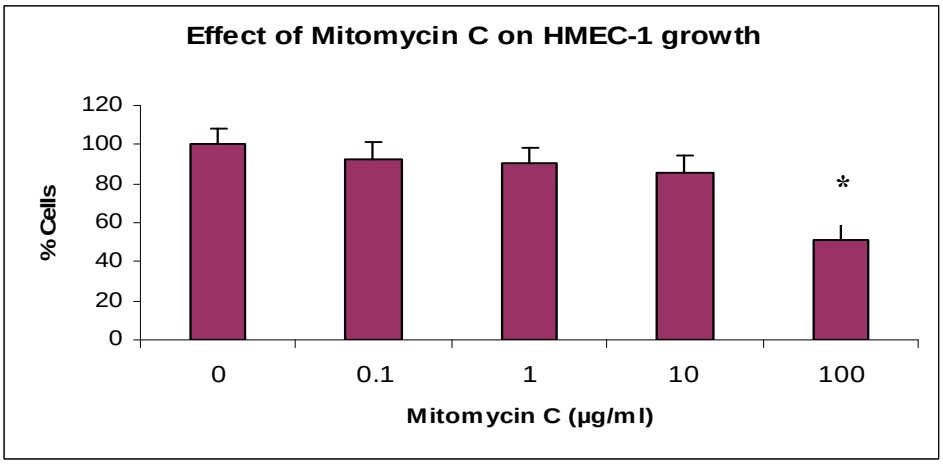


Figure 4.5. Effect of cytochalasin D on BME cell migration.
Confluent BME cell monolayers were wounded with a blade so as to mark an initial wound edge and washed. Cells were then treated with DMSO (control), cytochalasin D. After 15 hours, cultures were fixed and stained, and subsequently viewed with a light microscope. Within the wound, seven fields measuring 1.0 x 1.4mm were selected. The total number of cells in each field was determined.

4.4.2. Endothelial Cell Growth

The effects of the various test drugs on endothelial cell growth were initially determined using HMEC-1. Cells were exposed to various concentrations of bleomycin, mitomycin C, 2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine and nocodazole, and subsequently stained with crystal violet stain. The number of viable cells was then determined. Results represent the number of viable cells as a percentage of control cells calculated from the mean of three wells. Experiments were done in triplicate. Bleomycin caused a slight reduction in cell numbers, but had no statistically significant effect on this cell line. Mitomycin C also caused a slight reduction in cell numbers. Although mitomycin C resulted in a significant reduction in cell number at doses of 100 µg/ml, 50% inhibition of cell growth was not attained with the drug.

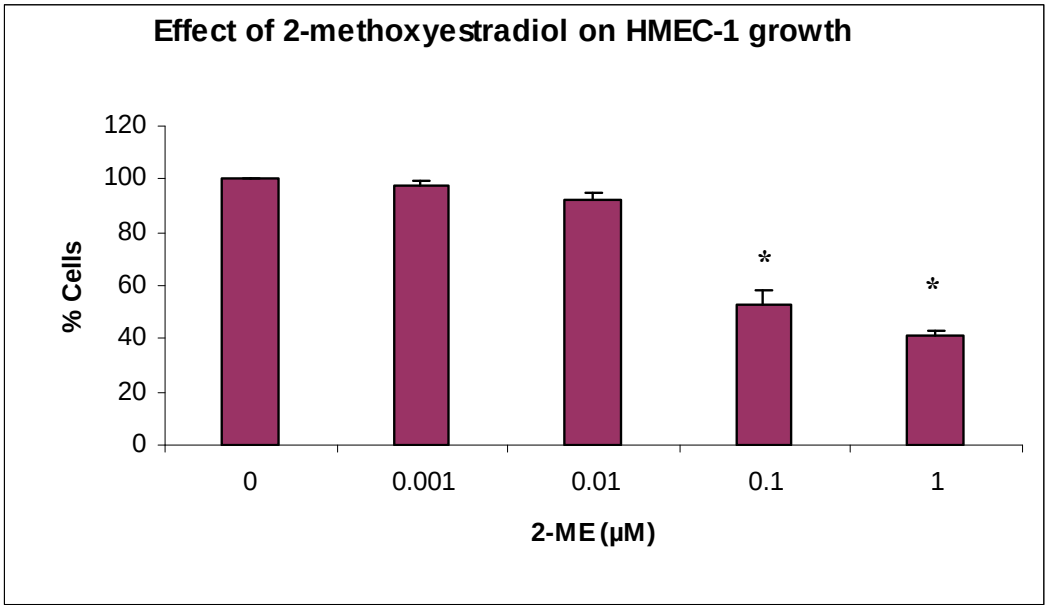


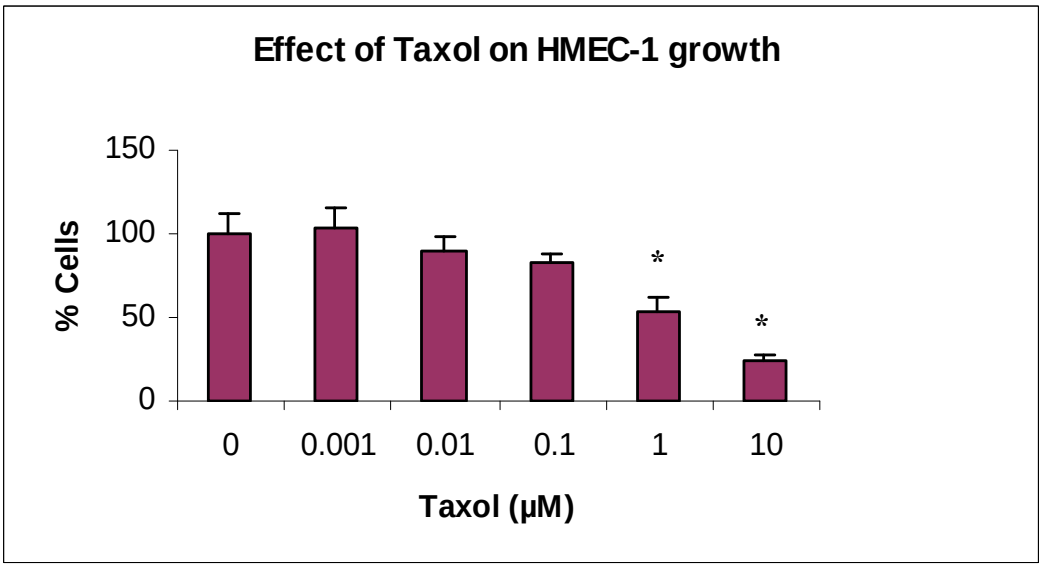


* Significant difference between the number of control and treated cells: $P < 0.05$

Figure 4.6. Effects of bleomycin and mitomycin C on HMEC-1 growth.
Cells were seeded at a density of 20000 cells per well in 24-well culture plates and exposed to bleomycin/mitomycin C or saline. After 48 hours cultures were fixed in 1% glutaraldehyde and stained with 1% crystal violet. The chromophore was extracted with 0.2% triton X-100, and the absorbance was read at 570 nm. Results represent the mean ± SD. Three wells were analysed for each concentration. Data shown are representative of three independent experiments.

2-ME induced a significant decrease in the percentage of viable HMEC-1 at doses of 0.1 µM and above. Taxol caused a significant decrease in cell numbers at a dose of 1 µM, and a maximum decrease in cell numbers was observed at 10 µM.

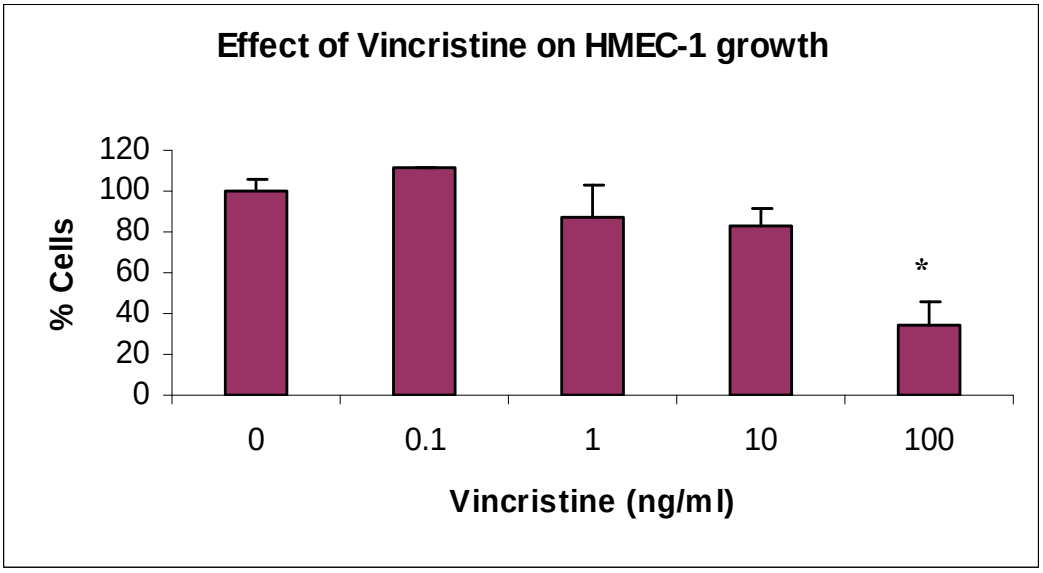


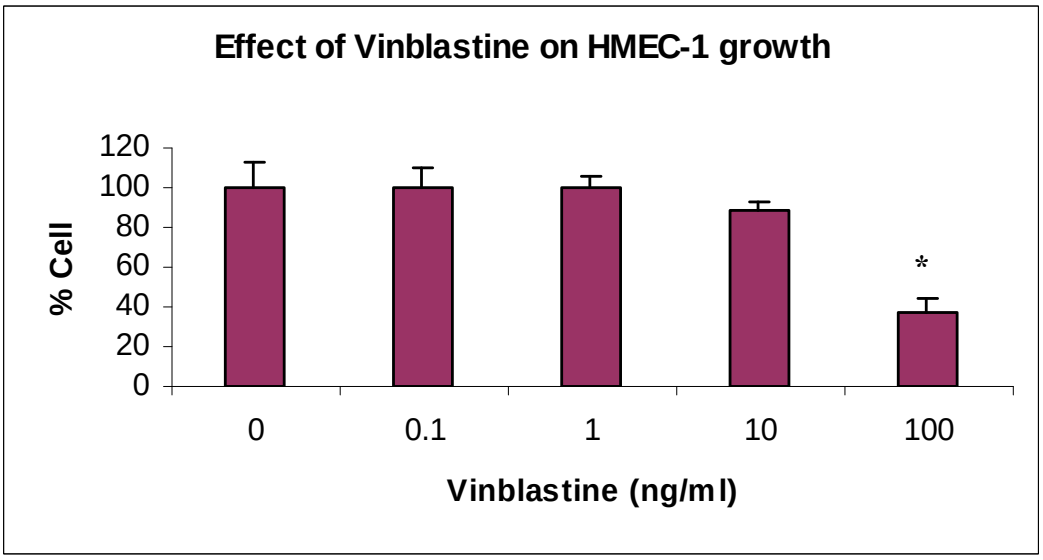


* Significant difference between the number of control and treated cells: $P < 0.05$

Figure 4.7. Effects of 2-methoxyestradiol and taxol on HMEC-1 growth.
Cells were seeded at a density of 20000 cells per well in 24-well culture plates and exposed to 2-ME/taxol or DMSO. After 48 hours cultures were fixed in 1% glutaraldehyde and stained with 1% crystal violet. The chromophore was extracted with 0.2% triton X-100, and the absorbance was read at 570 nm. Results represent the mean ± SD. Three wells were analysed for each concentration. Data shown are representative of three independent experiments.

At lower doses (0.1 to 10 ng/ml), neither vincristine nor vinblastine had an effect on endothelial cell numbers. Both drugs induced significant inhibition of HMEC-1 growth at a dose of 100 ng/ml.

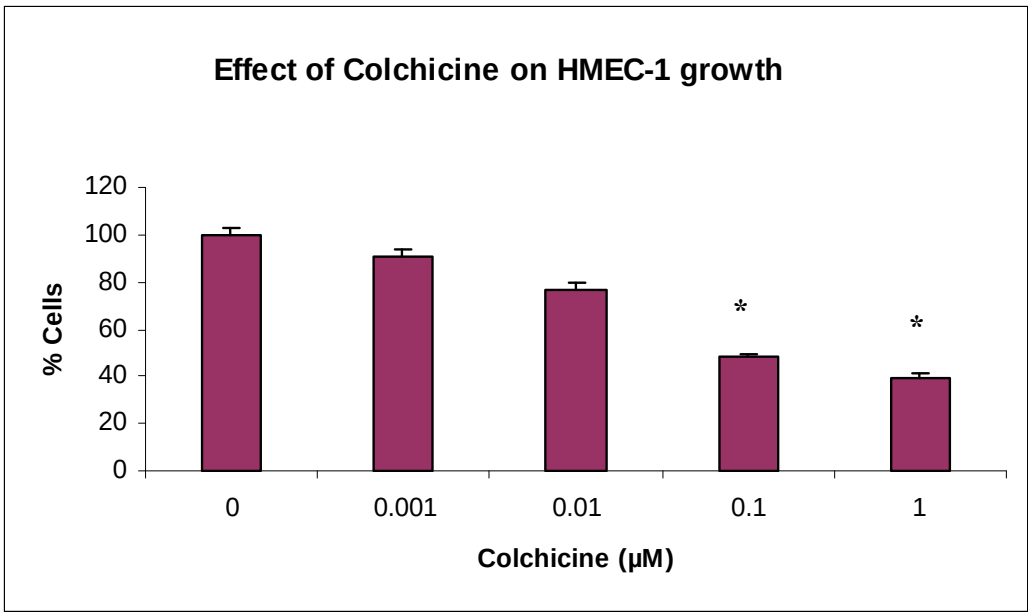


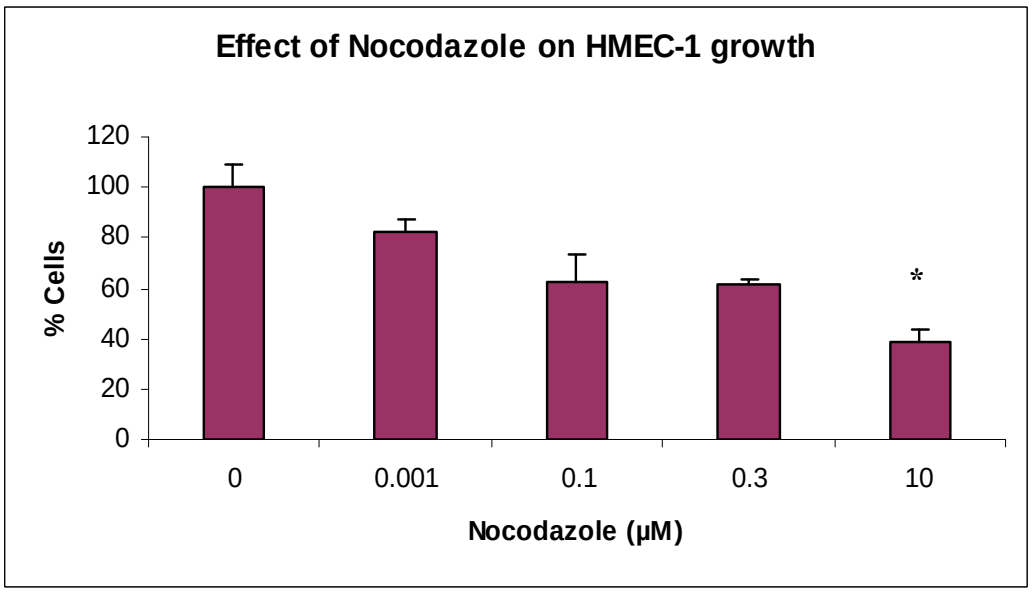


* Significant difference between the number of control and treated cells: $P < 0.05$

Figure 4.8. Effects of vincristine and vinblastine on HMEC-1 growth.
Cells were seeded at a density of 20000 cells per well in 24-well culture plates and exposed to vincristine/vinblastine or saline. After 48 hours cultures were fixed in 1% glutaraldehyde and stained with 1% crystal violet. The chromophore was extracted with 0.2% triton X-100, and the absorbance was read at 570 nm. Results represent the mean ± SD. Three wells were analysed for each concentration. Data shown are representative of three independent experiments.

Both colchicine and nocodazole had a dose-dependent inhibitory effect on HMEC-1 growth, with IC_{50} values ranging of 0.095-0.32 µM respectively. Colchicine caused a slight decrease in cell numbers, at concentrations of 0.001 and 0.01 µM. A significant decrease in cell numbers was observed following exposure to 0.1 µM colchicine and 10 µM nocodazole.

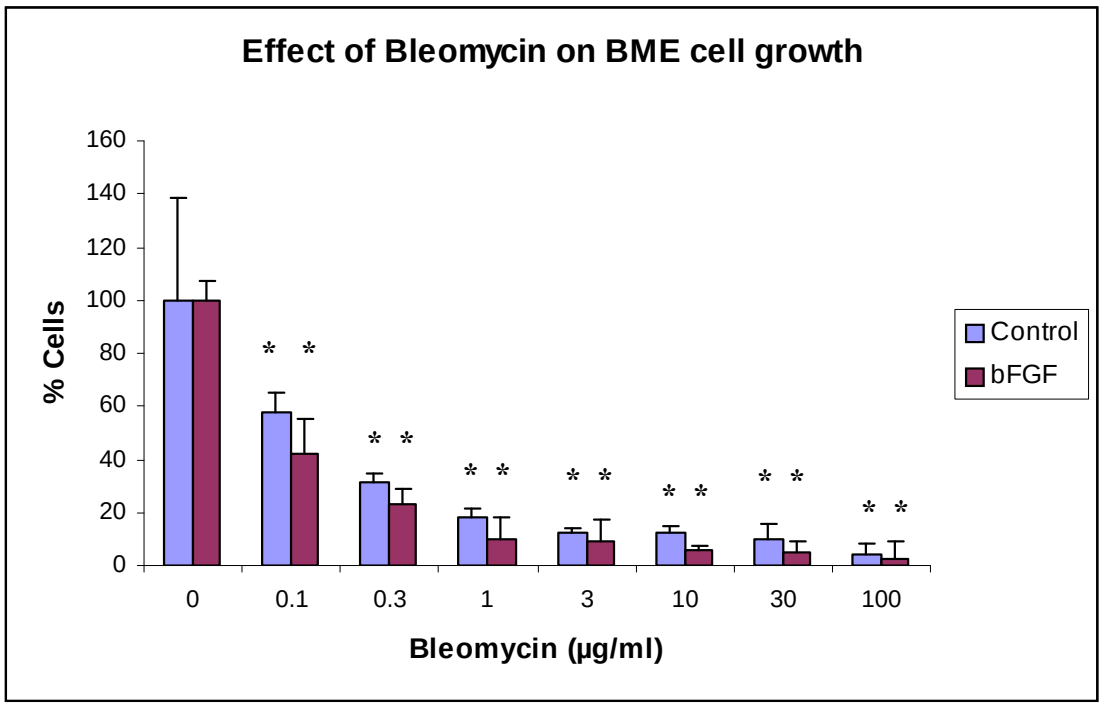


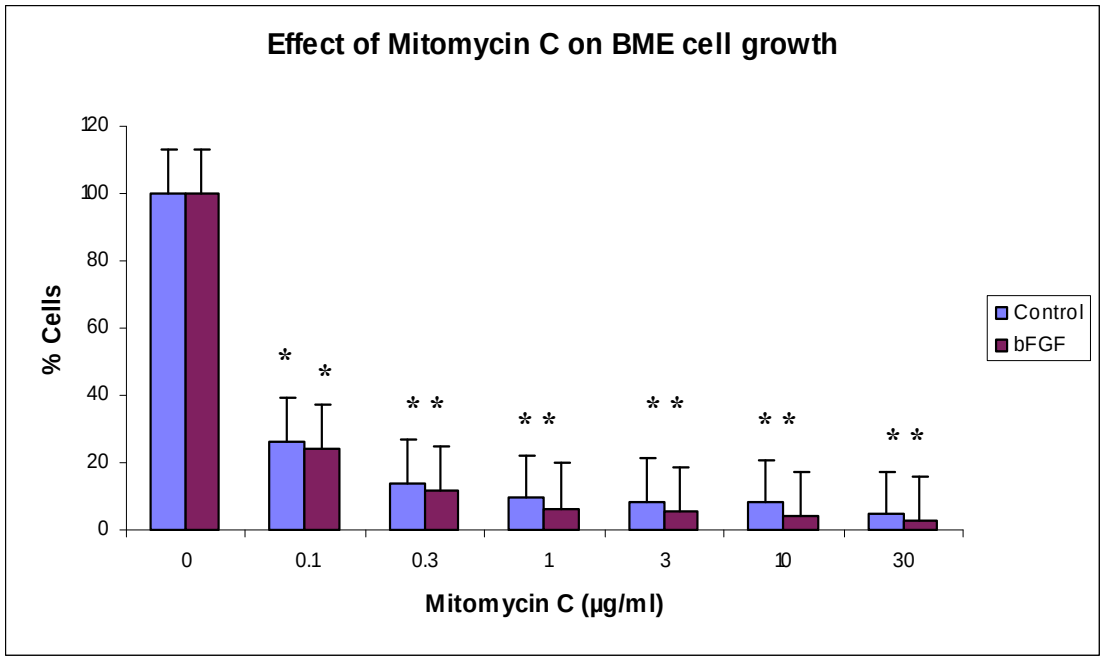


* Significant difference between the number of control and treated cells: $P < 0.05$

Figure 4.9. Effects of colchicine and nocodazole on HMEC-1 growth.
Cells were seeded at a density of 20000 cells per well in 24-well culture plates and exposed to colchicine/nocodazole or DMSO. After 48 hours cultures were fixed in 1% glutaraldehyde and stained with 1% crystal violet. The chromophore was extracted with 0.2% triton X-100, and the absorbance was read at 570 nm. Results represent the mean ± SD. Three wells were analysed for each concentration. Data shown are representative of three independent experiments.

Further studies on the effects of test drugs on endothelial cell growth were undertaken using BME cells. Bleomycin and mitomycin C inhibited BME cell growth, with IC_{50} values of 0.158 µg/ml (0.105 µM) and 0.05 µg/ml (0.149 µM) respectively (table 4.1). The two drugs also inhibited bFGF-induced BME cell growth, with the inhibitory effect being significant from 0.1 µg/ml for both drugs.

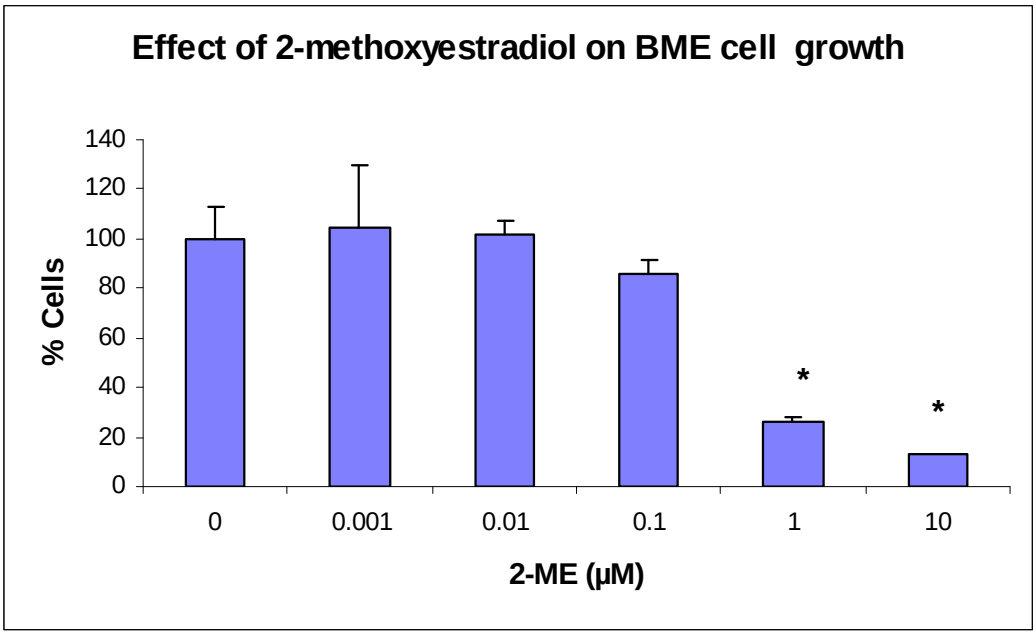


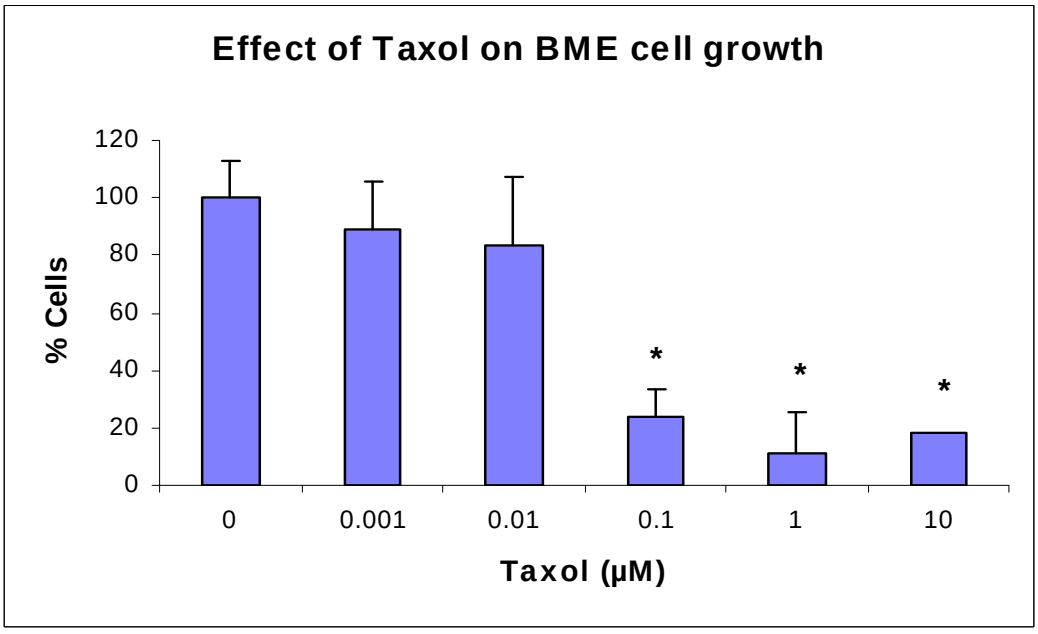


* Significant difference between the number of control and treated cells: $P < 0.05$

Figure 4.10. Effects of bleomycin and mitomycin C on BME cell growth.
Cells were seeded at a density of 10000 cells per well in 24-well culture plates and exposed to bleomycin/mitomycin C or saline. Cells were subsequently counted using a FACScan analyser. Results represent the mean ± SEM. Three wells were analysed for each concentration. Data shown are representative of least two independent experiments.

The level of 2-ME cytotoxicity was less than that seen with taxol. Significant inhibitory effect following exposure to 2-ME occurred at a dose of 1 µM, while taxol exerted a significant effect at a dose of 0.1 µM. The effects of 2-ME and taxol on bFGF-induced cell growth are listed in table 4.2.

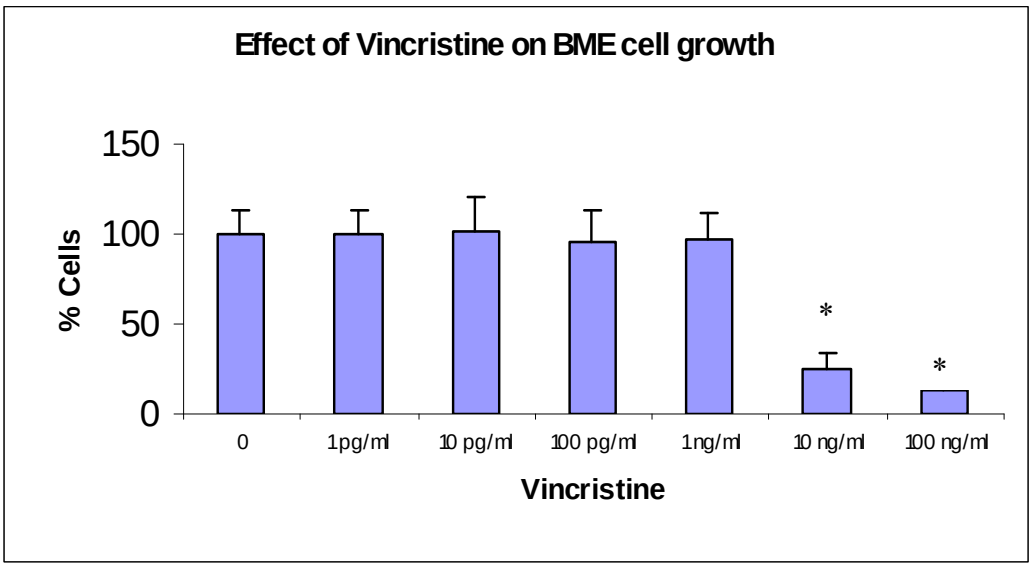


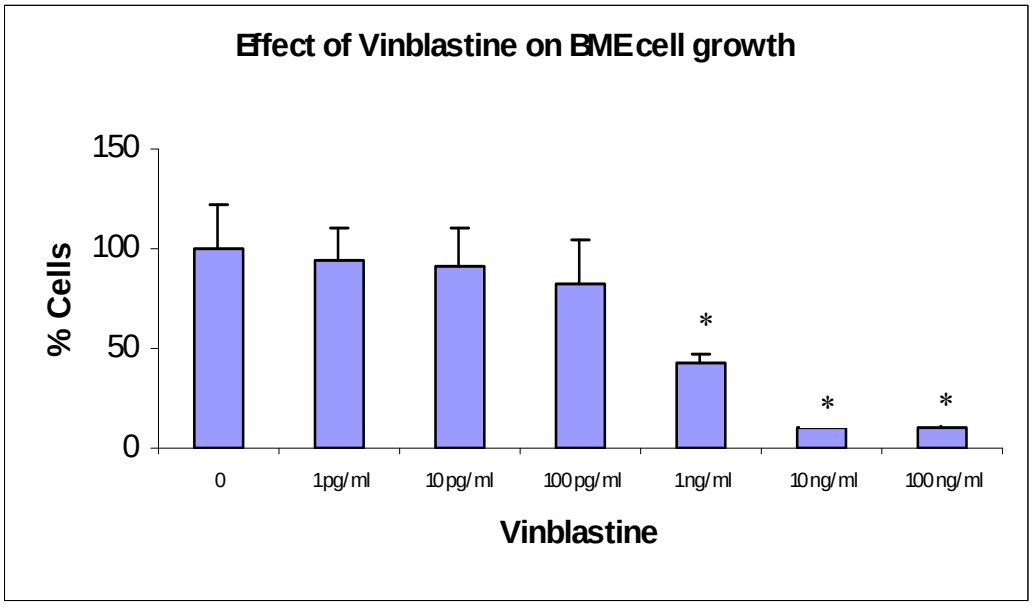


* Significant difference between the number of control and treated cells: $P < 0.05$

Figure 4.11. Effects of 2-methoxyestradiol and taxol on BME cell growth.
Cells were seeded at a density of 10000 cells per well in 24-well culture plates and exposed to 2ME/taxol or DMSO. Cells were subsequently counted using a FACScan analyser. Results represent the mean ± SEM. Three wells were analysed for each concentration. Data shown are representative of least two independent experiments.

Vincristine and vinblastine inhibited growth of endothelial cells in a dose-dependent manner, however, vinblastine was more potent as shown by the IC_{50} values of 3.98 ng/ml (0.004 µM) and 0.63 ng/ml (0.690 nM) respectively (table 4.1).. The effects of vinca alkaloids on bFGF-induced cell growth are listed in table 4.2.

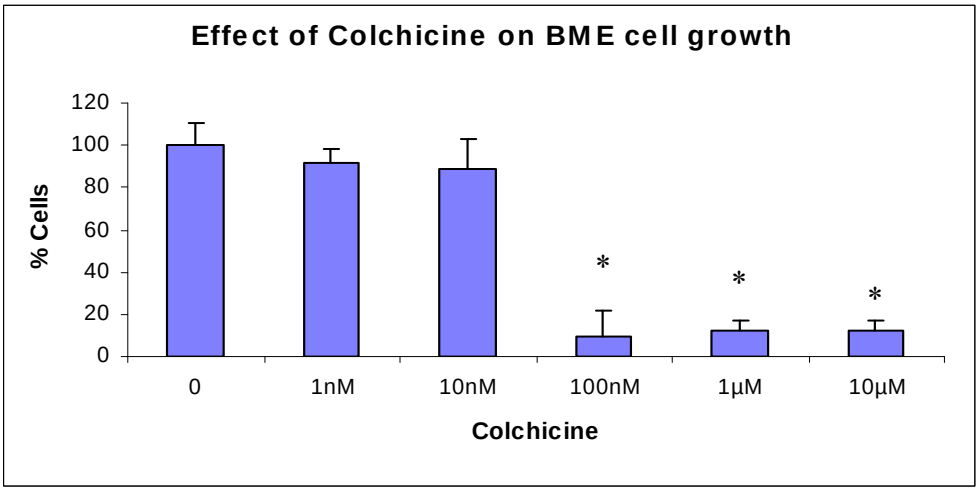


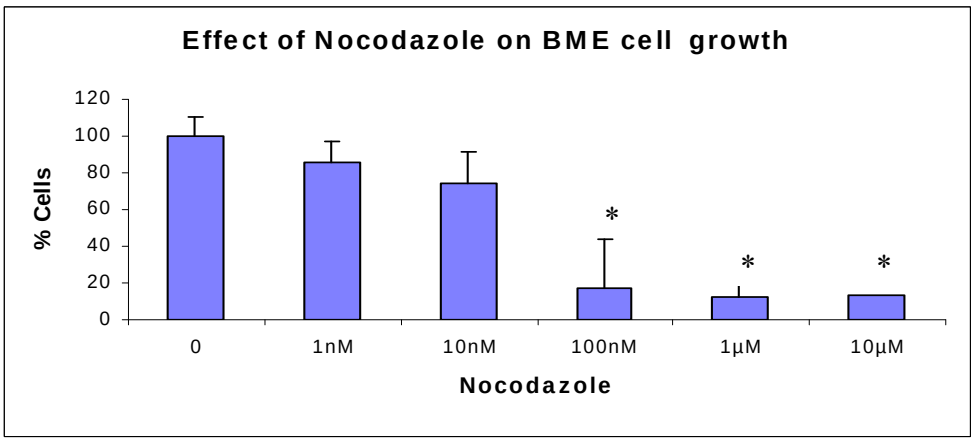


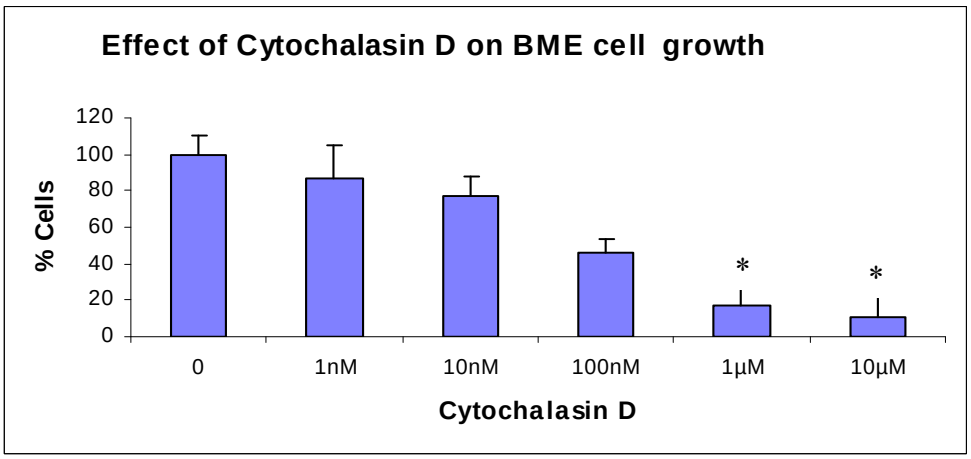
* Significant difference between the number of control and treated cells: $P < 0.05$

Figure 4.12. Effects of vincristine and vinblastine on BME cell growth.
Cells were seeded at a density of 10000 cells per well in 24-well culture plates and exposed to vincristine/vinblastine or saline. Cells were subsequently counted using a FACScan analyser. Results represent the mean ± SEM. Three wells were analysed for each concentration. Data shown are representative of least two independent experiments.

Colchicine induced a dose-dependant inhibition of BME cell growth with an IC_{50} of 0.032 µM. The effect of colchicine on BME cell growth were mimicked by nocodazole (IC_{50} 0.031 µM). Cytochalasin D induced a significant decrease in cell numbers at doses of 1 µM and above. The effects of drugs on bFGF-induced cell growth are listed in table 4.2.







* Significant difference between the number of control and treated cells: $P < 0.05$

Figure 4.13. Effects of colchicine, nocodazole and cytochalasin D on BME cell growth. Cells were seeded at a density of 10000 cells per well in 24-well culture plates and exposed to of colchicine/nocodazole/cytochalasin D or DMSO. Cells were subsequently counted using a FACScan analyser. Results represent the mean ± SEM. Three wells were analysed for each concentration. Data shown are representative of least two independent experiments.

Table 4.1. The effects of test drugs on endothelial cell growth. The IC_{50} value for each drug was calculated as the concentration of test drug resulting in a 50% reduction of viable cells compared to untreated cells.

Bleomycin	HMEC-1	ND	BME cells	0.105 µM (0.158 µg/ml)
Mitomycin C	HMEC-1	ND	BME cells	0.149 µM (0.050 µg/ml)
2-methoxyestradiol	HMEC-1	0.250 µM	BME cells	0.398 µM
Taxol	HMEC-1	0.977 µM	BME cells	0.040 µM
Vincristine	HMEC-1	0.068 µM (63.09 ng/ml)	BME cells	0.004 µM (3.980 ng/ml)
Vinblastine	HMEC-1	0.044 µM (39.81 ng/ml)	BME cells	0.690 nM (0.630 ng/ml)
Colchicine	HMEC-1	0.095 µM	BME cells	0.032 µM
Nocodazole	HMEC-1	0.320 µM	BME cells	0.031 µM
Cytochalasin D	HMEC-1	ND	BME cells	0.063 µM

ND – Not Determined

Bleomycin inhibited BME cell growth with an IC_{50} of 0.105 µM. Bleomycin appeared more effective in inhibiting call growth induced by bFGF (table 4.2). Cytoskeletal-disrupting drugs, 2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine and nocodazole induced 50% inhibition of cell growth at much lower doses than bleomycin and mitomycin C. However, vincristine and vinblastine were the most potent in inducing a reduction in BME cell numbers (table 4.1). The IC_{50} value for vincristine was the same in the presence and absence of bFGF.

Table 4.2. The effects of test drugs on the growth of endothelial cells treated with 10 ng/ml basic fibroblast growth factor (bFGF). Results are expressed as IC_{50} values.

Drug	**Cell Type**	**IC_{50}**
Bleomycin	BME cells	0.053 µM (0.080 µg/ml)
Mitomycin C	BME cells	0.180 µM (0.060 µg/ml)
2-methoxyestradiol	BME cells	0.018 µM
Taxol	BME cells	0.063 µM
Vincristine	BME cells	0.004 µM (3.981 ng/ml)
Vinblastine	BME cells	0.900 nM (0.891 ng/ml)
Colchicine	BME cells	0.017 µM
Nocodazole	BME cells	0.022 µM
Cytochalasin D	BME cells	ND

ND – No 50 % inhibition of cell growth was observed.
IC_{50} was not attained with Cytochalasin D at doses used in this study. A 52.55 % reduction in cell number was observed following exposure of cells to the highest dose of 100 nM.

4.5. Discussion

Angiogenesis is dependent on precisely controlled sequential alterations in a number of endothelial cell functions which include migration and proliferation. [7] In this chapter, the effects of bleomycin, mitomycin C, 2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole, and cytochalasin D on these cell functions were determined.

With respect to endothelial cell migration, it was found that all drugs, with the exception of mitomycin C, inhibited BME cell migration with varying potency. Previous studies have shown that the microtubule-stabilizing drug, taxol inhibits endothelial cell migration and angiogenesis *in vitro*. [15] 2- methoxyestradiol has also been shown to have an anti-migratory effect on bovine brain capillary endothelial cells and BME cells. [16] Both taxol and 2-ME were also shown to inhibit neovascularization in mice. [17]

The effects of test drugs on human microvascular endothelial cell (HMEC-1) growth were investigated using the crystal violet nuclear staining assay. Bleomycin and mitomycin C caused marginal decreases in human microvascular endothelial cell numbers, while cytoskeletal-disrupting agents (2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine and nocodazole) induced a significant decrease in HMEC-1. Unexpectedly, bleomycin, the main drug of interest in this study, had no statistically significant effect on HMEC-1 growth, it was therefore decided to evaluate the effects of this drug on another endothelial cell line, bovine microvascular endothelial (BME) cells.

Preliminary studies on the effects of bleomycin on BME cells revealed a dose-dependent inhibition of cell growth (results not shown). However, BME cells appear to attach loosely to the surface of culture flasks. Indeed, when floating BME cells were harvested and stained with trypan blue, these cells appeared viable under the light microscope. Since loosely attached cells can be lost during the rinsing stages of the crystal violet nuclear staining technique, decreased cell adherence could be interpreted as growth inhibition.

Therefore, further studies on the effects of bleomycin, mitomycin C, 2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole and cytochalasin D on BME cell growth were conducted using a FACScan Analyser. With this technique, floating and adherent cells were pooled together and analysed.

Bleomycin induced a dose-dependent inhibition on BME cell growth. In this study bleomycin had no effect on HMEC-1 growth, while previous studies revealed relative resistance of HMEC-1 to the drug, with an IC_{50} value of 106 µg/ml being reported. [18]

The reason for the difference in cytotoxic effects of bleomycin in the two cell lines is not known. However, chromatographic studies conducted in our laboratory indicated that there might be limited uptake of bleomycin by HMEC-1 (data not shown). In a previous report, the effects of bleomycin on HMEC-1 were enhanced by increasing the number of internalized drug molecules through the application of an electric current to the cells.

Bleomycin induced a significant decrease in cell numbers in bFGF-treated BME cells. Mitomycin C, another antineoplastic antibiotic, also caused a significant decrease in BME cell numbers in the presence and absence of bFGF, possibly due to inhibition of DNA synthesis. Previously, mitomycin C was shown to inhibit DNA synthesis and cause cell cycle arrest in endothelial cells. [19] Several growth factors have been reported to affect one or more endothelial cell functions involved in the process of angiogenesis, the best characterized being basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). [11] Both bFGF and VEGF have been reported to induce the proliferation and migration of endothelial cells. [11,14] The ability of these test drugs to inhibit cell growth in the presence of VEGF and bFGF may thus have clinical importance.

In accordance with previous studies,[15,16] it was observed in this study that taxol and 2-ME inhibited endothelial cell proliferation in a dose-dependent manner. Previous reports suggest that abnormal microtubule assembly might be responsible for 2-ME effects on proliferating cells. [11] Furthermore, both 2-ME and taxol also inhibited BME cell growth stimulated by bFGF, although with more potency than DNA-damaging drugs.

The vinca alkaloids, vincristine and vinblastine, were the most potent of all tested drugs in inhibiting BME cell growth, with IC_{50} values of 0.004 and 0.690 nM respectively. In a previous study, vinblastine was shown to reversibly inhibit endothelial cell growth, although at much lower doses than those employed in this study (0.25 pM). [20] Colchicine, nocodazole and Cytochalasin D caused a reduction in BME cell numbers in a dose-dependent manner, with IC_{50} values ranging between 0.031-0.063 µM.

In a previous study, disruption of microfilaments by cytochalasin D lead to inhibition of cell growth, while microtubule-disruption by nocodazole induced partial inhibition of endothelial cell growth. [8] According to Ingber *et al*. (1995) actin filaments and microtubules play important roles in endothelial cell growth during angiogenesis. [8]

Data presented in this chapter demonstrated that test drugs (1) inhibited endothelial cell migration (with the exception of mitomycin C) and (2) endothelial cell growth. These findings therefore suggest that test drugs may inhibit angiogenesis through inhibition of either endothelial cell migration and/or endothelial cell growth. Since excessive angiogenesis has been implicated in the development of haemangiomas, the potential antiangiogenic effects of these test drugs warrant further investigation.

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CHAPTER 5

Bleomycin, mitomycin C, and cytoskeletal-disrupting agents induce apoptosis in bovine microvascular endothelial cells

5.1. Introduction

A balance between endothelial cell growth and apoptosis is crucial for the maintenance of homeostasis of the vascular endothelial cell population.[1,2] Angiogenic growth factors, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) play a role in the maintenance of endothelial cell survival and inhibit EC apoptosis by modulating gene expression, and through post-transcriptional regulation of anti-apoptotic protein kinases (Fig 5.1). [2,3,4] Studies have shown that deprivation of these growth factors leads to apoptosis of endothelial cells. [1,2] Apoptosis is an energy-dependent process in which living cells participate in their own death in an organised manner, and which involves the activation of a group of cysteine proteases called caspases. [5-7]

Apoptosis is characterised by a number of morphological and biochemical alterations in cells. [6,8] The morphological changes associated with apoptosis include cell shrinkage and budding of the plasma membrane. In the nucleus, initially, chromatin condensation occurs around the periphery, and the nuclear laminar begins to disappear. [8] Later in the process, highly condensed chromatin with cleaved DNA segregates into defined structures which have an intact nuclear envelope. [7,8] Eventually, the cell breaks up into many spherical bodies called apoptotic bodies, which contain compacted organelles and/or nuclear material. These bodies are mostly engulfed by neighbouring cells, in particular macrophages.[7,8] Biochemical determinants of apoptosis include the formation of internucleosomal DNA fragments of 180 to 200 base pairs.[8,9]

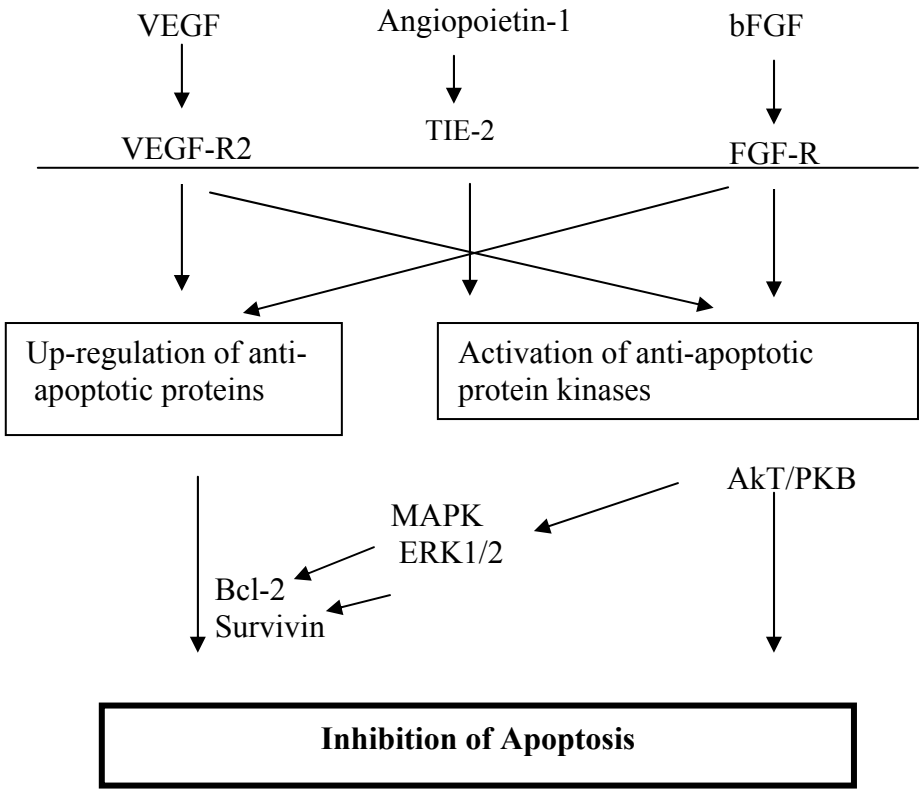

Figure 5.1. Regulation of endothelial cell apoptosis by angiogenic factors.

Adapted from Chavakis and Dimmeler Arterioscler. Thromb Vasc Biol 2002; 22: 887-893

According to Elmore (2007), microscopic observation of morphological changes combined with DNA fragmentation assays are two reliable independent techniques that define cell death occurring by apoptosis. [9]

In this chapter the effects of test drugs on endothelial cell morphology were determined using light microscopy (because of the simplicity and cost-effectiveness of the technique). The potential apoptotic effects of test drugs were further assessed using an assay of DNA fragmentation and acridine orange staining.

5.2. Materials and Method

5.2.1. Cell Morphology

Viable BME cells were seeded aseptically onto heat-sterilised coverslips in six-well culture plates at a density of 2 x 10^5 cells per well. Cells were exposed to bleomycin, mitomycin C, 2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole, or to drug vehicles for 48 hours. The vehicle for bleomycin, mitomycin C, and the vinca alkaloids was saline, while the vehicle for 2-ME, taxol, colchicine, nocodazole and cytochalasin D was 0.05% DMSO. At termination, coverslips were inserted into staining dishes and cells were fixed in Bouin's fixative for 30 minutes. Cells were then left in 70% ethanol for 20 minutes and then rinsed with tap water. Thereafter, cells were stained with Mayer's haemalum for 15 minutes (twice), rinsed with tap water, and subjected to 1% eosin for two minutes. Cells were then rinsed for five minutes in each of the following solvents: 70%, 90%, 100% ethanol and xylol. Finally, coverslips were mounted onto microscope slides with mounting resin. A Nikon Optiphot light microscope was used to study cell morphology and photos were taken using a Nikon digital camera.

5.2.2. DNA fragmentation

Bovine microvascular endothelial cells were seeded into 60 mm tissue culture dishes at a density of 1x10^6 cells/plate. After 24 hours cells were exposed to bleomycin, mitomycin C, 2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole, and cytochalasin D or vehicles for 48 hours. At the end of the incubation, floating cells were recovered from culture media by centrifugation, and adherent cells were washed twice with PBS. Floating and adherent cells were lysed together in 250 µl/tissue culture dish containing 20 mM Tris-HCl (pH 7.4), 5 mM EDTA (pH 7.4), 0.4% Triton X-100. Cell lysates were centrifuged at 13,000 rpm for 30 minutes at 4°C to pellet nuclei.

Supernatants were extracted with equal volumes of phenol-chloroform, precipitated with 0.5 vol. of 7.5 M ammonium acetate (pH 7.5), and three volumes of ethanol, and then centrifuged at 13,000 rpm for 30 minutes at 4°C. Samples were left overnight at -20°C. Pellets were resuspended in 10 mM Tris-HCl (pH 8.0),1 mM EDTA (pH 8.0), containing DNase-free pancreatic ribonuclease (20 µg/ml), incubated at 37°C for 30 minutes and loaded on to 1.8% agarose gels containing ethidium bromide (1 µg/ml). [1,10]

Samples were then electrophoresed at 10 V/cm for two hours. DNA was visualised by UV fluorescence. The appearance of ladder bands signifies DNA cleavage, one of the hallmarks of apoptosis.

5.2.3. Acridine-Orange staining

The mode by which the test drugs induced cell death was further assessed using acridine orange (AO) staining. Cells were seeded in 34.6 mm diameter wells at a density of 200 000 cells per well. After 24 hours, BME cells were grown in the presence of the various drug preparations/vehicles for 48 hours.

Cells were harvested as follows: medium and floating cells were transferred to 15 ml tubes. The rest of the adherent cells were detached with 0.25% trypsin-1mM EDTA. The floating and detached cells from the same sample were pooled together in 15 ml tubes, pelleted by centrifugation at 1000 rpm for 5 minutes using a Beckman Model centrifuge, and cell pellets were washed with 1 ml PBS, and subsequently re-suspended in 25 µl PBS. Cells were then incubated with 10 µg/ml acridine orange for 5 minutes. Stained cell suspensions (10 µl) were placed on microscope slides and covered with coverslips. [11,12]

Cells were viewed and counted using a Zeiss Axiovert inverted microscope at 400X magnification. Pictures were taken with a Nikon digital camera. Apoptosis was identified as nuclear chromatin condensation. Randomly selected microscopic fields were examined for this experimental condition, and the percentage of cells undergoing apoptosis in each field was determined. Results are expressed as the mean percentage (± SD) of cells with evidence of apoptosis. Tests were done in triplicate.

5.3. Results

5.3.1. Cell Morphology

Hematoxylin and eosin (H&E) staining of bleomycin treated-BME cells was undertaken to determine the effects of the drug on cell morphology. In control cultures, cells in interphase and telophase and were observed (fig 5.2 A;B).

Condensed chromatin and structures that resembled apoptotic bodies were evident in bleomycin, mitomycin C, colchicine, nocodazole, and taxol treated cultures. In 2-ME treated cultures, cells in metaphase were observed, however, only some of the chromosomes in these cells were aligned along the equator, while other chromosomes were aligned closer to the poles (fig 5.2 F). Cells which appeared round with dense nuclear chromatin fragments were also observed in 2-ME treated cultures, and according to Elmore (2007), such morphologic appearance is a characteristic of apoptosis. [9]

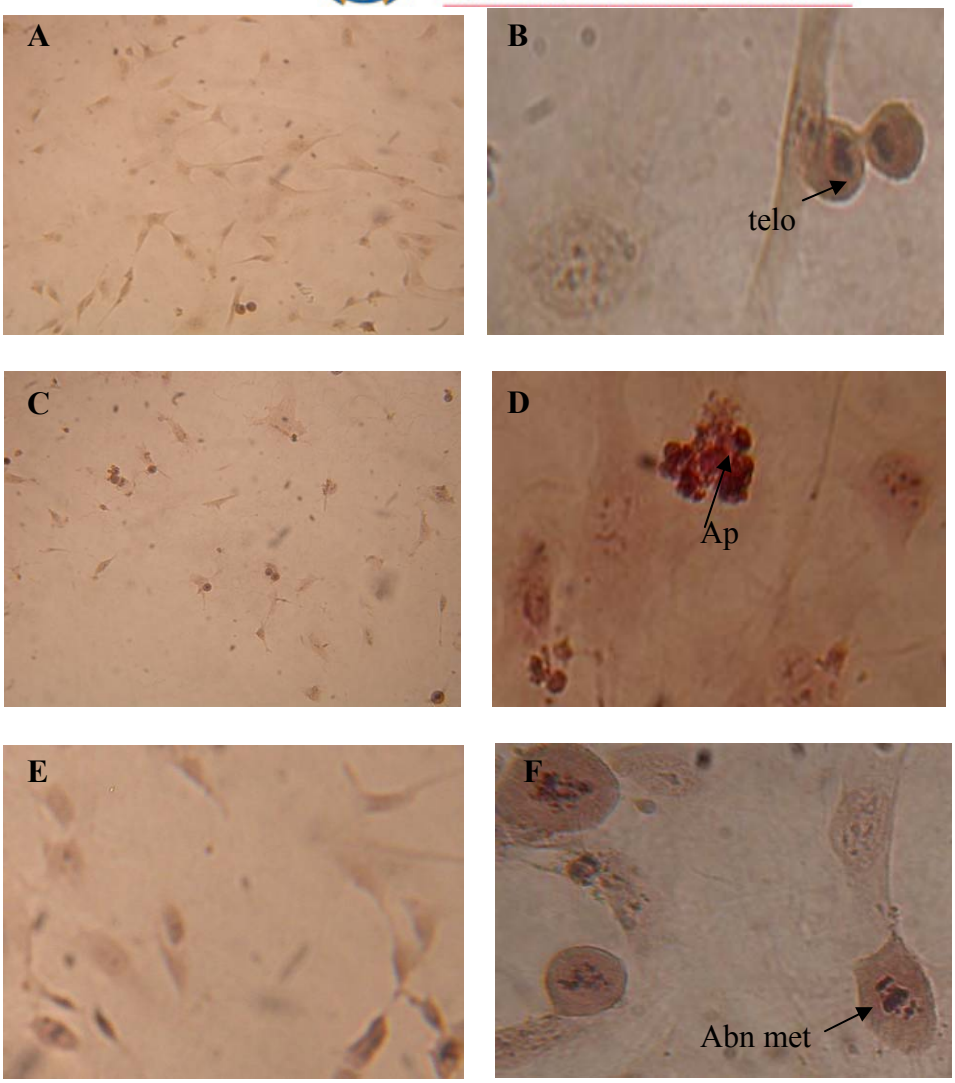

Figure 5.2. Photomicrographs of haematoxylin and eosin (H&E) stained BME cells after 48 hours of exposure to bleomycin or 2-ME.

A and B: Control cells were exposed to vehicle (DMSO) only. Most cells were in interphase, cells in telophase were also observed (arrow). C and D: Cells exposed to bleomycin. The arrow indicates an apoptotic cell. E and F: Cells exposed to 2-ME, the arrow indicates a cell undergoing abnormal metaphase. (A, C, E: original magnification 100X; B, D, F: original magnification 400X)

Ap = apoptosis; Abn met = abnormal metaphase; telo = telophase

5.3.2. DNA Fragmentation

Bleomycin **Mitomycin C**

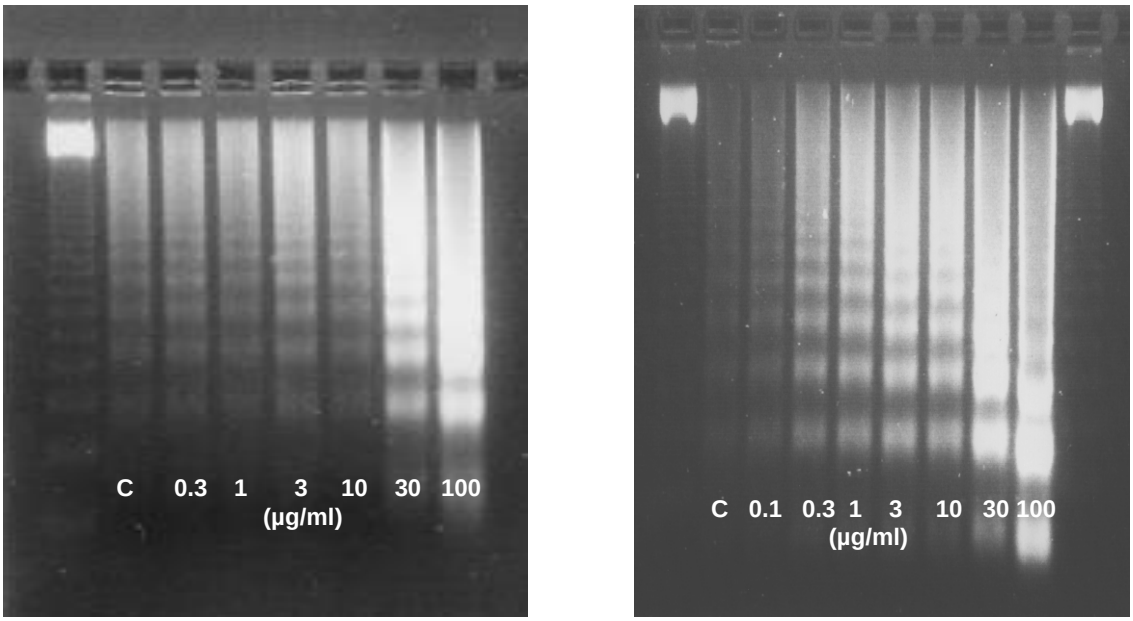

Figure 5.3. Analysis of internucleosomal fragmentation in DNA preparations following exposure of BME cells to bleomycin and mitomycin C.

Bleomycin induced DNA strand breaks in a dose-dependent manner (fig 5.3). The most intense ladders were observed following exposure to 30 and 100 µg/ml of bleomycin. DNA strand breaks were also observed in samples exposed to mitomycin C at doses of 0.3 µg/ml and above (fig 5.3). The intensity of the ladders increased with increasing drug concentration.

With respect to cytoskeletal-disrupting drugs, internucleosomal DNA fragmentation was detected only after exposure to the highest dose of 2-ME (10 µM), while taxol and colchicine induced DNA strand breaks at doses of 100 nM and above (fig 5.4). However, no difference in intensity was observed between samples exposed to 1 and 10 µM of taxol. In the case of cytochalasin D treated cells, internucleosomal strand breaks were observed following exposure to doses of 1 and 10 µM, while fragmentation of DNA was detected at 1 and 10 µM in nocodazole-treated cells, and again, increasing drug concentration did not result in increased intensity of DNA strands. Results of vincristine and vinblastine treated cultures were inconclusive.

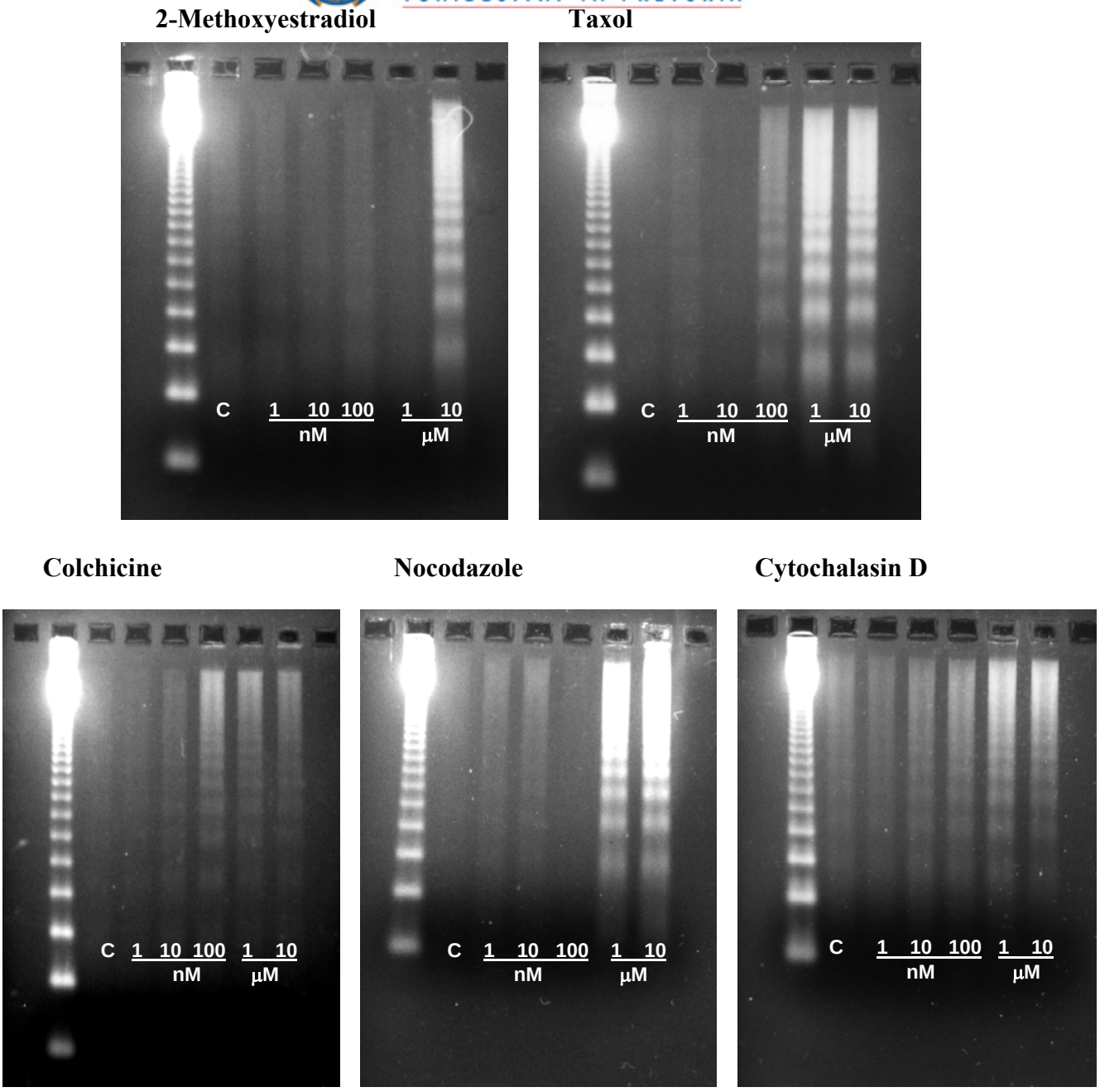

Figure 5.4. Analysis of internucleosomal fragmentation in DNA preparations following exposure to cytoskeletal-disrupting drugs. DNA isolated from untreated preparations was compared to DNA from cells exposed to varying concentrations of the different drugs.

5.3.3. Acridine Orange Staining

Drug-induced apoptosis was confirmed by endothelial cell nuclear acridine orange incorporation. Bovine microvascular endothelial cells were incubated with bleomycin, mitomycin C, 2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole and cytochalasin D for 48 hours, and AO staining was subsequently performed. Table 5.1 shows levels of apoptosis as determined by AO labelling of BME cells treated with the various test drugs. Bleomycin increased AO staining of BME cell nuclei (fig 5.5 B) when compared to the control (fig 5.5 A). Increased AO staining of BME cell nuclei was also observed in cultures treated with mitomycin C, -methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole and cytochalasin D (table 5.1).

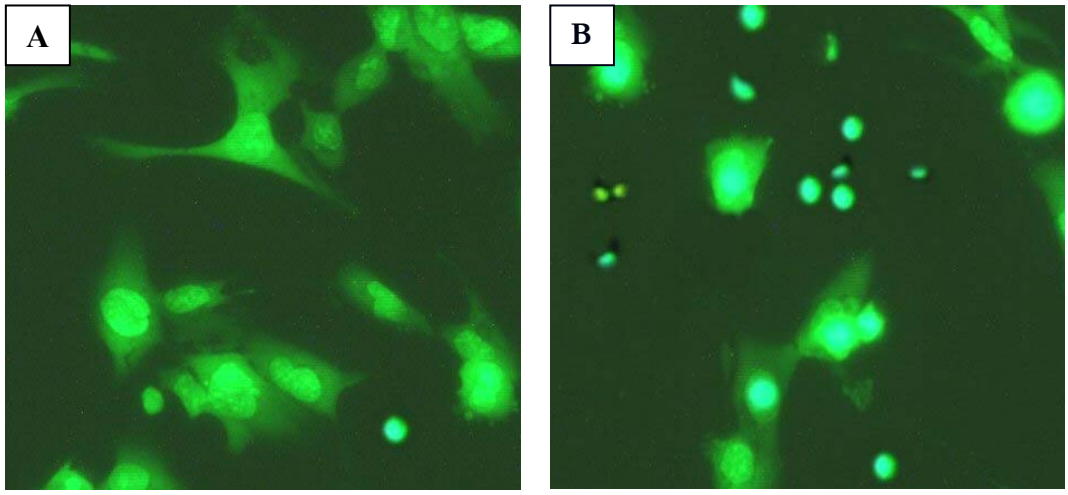

Figure 5.5. Micrographs of acridine orange (AO) stained BME cells after 48 hours of exposure to (A)- vehicle (saline) or (B)-bleomycin. Magnification x400

Table 5.1. Test drugs induce apoptosis in BME cells

Control (Saline)	5.92 ± 4.28
(DMSO)	7.35± 11.25
Bleomycin	
0.1 µg/ml	11.26 ± 9.62
1 µg/ml	18.53 ± 11.65*
10 µg/ml	21.03 ± 11.61*
100 µg/ml	41.72 ± 16.35*
Mitomycin	
0.1 µg/ml	22.55 ± 18.6*
1 µg/ml	33.76 ± 18.21*
10 µg/ml	58.10 ± 20.42*
Colchicine	
0.01 µM	6.21 ± 4.02
0.1 µM	10.25 ± 12.05
1 µM	33.52 ± 11.26*
10 µM	31.05 ± 9.65*
Nocodazole	
0.01 µM	12.53 ± 9.36
0.1 µM	21.4 ± 12.22*
1 µM	43.87 ± 9.75*
10 µM	40.25 ± 8.15*
2-ME	
0.01 µM	18.57 ± 8.05
0.1 µM	14.23 ± 12.22
1 µM	32.81 ± 12.50*
10 µM	52.21 ± 10.02*
Taxol	
0.01 µM	17.82 ± 6.22
0.1 µM	28.74 ± 13.64*
1 µM	45.37 ± 10.80*
10 µM	44.00 ± 8.01*
Vincristine	
0.001 ng/ml	5.92 ± 6.10
0.1 ng/ml	5.87 ± 4.25
1 ng/ml	8.22 ± 9.65
10 ng/ml	27.26 ± 11.20*
Vinblastine	
0.001 ng/ml	6.04 ± 8.22
0.1 ng/ml	12.01 ± 8.75
1 ng/ml	10.72 ± 12.20
10 ng/ml	37.20 ± 11.26*

Confluent monolayers of BME cells were exposed to the agents indicated above, and apoptosis (acridine orange labelling) was determined after 48 hours. Values are the mean ± SD. Results are pooled from at least three separate experiments. An average of 12 fields was analyzed per treatment dose in each experiment; *Significant difference between control and drug-treated cells: $P < 0.05$. The control for bleomycin, mitomycin C, and vinca alkaloids was saline; the vehicle for other drugs was DMSO.

5.4. Discussion

The inhibition of apoptosis has been cited as an essential mechanism during angiogenesis. Apoptosis is a physiological process through which cells are eliminated from the body as a normal part of development and homeostasis. [2] The characteristic morphological and biochemical changes that define apoptosis have served as tools for the detection and analysis of this process. [9] To determine possible apoptosis induction by test drugs, effects of these drugs on cell morphology were investigated using light microscopy.

In control (DMSO-treated) cultures, normal cells in various stages of mitosis were observed (fig 5.2 B). Similar observations were made for cells treated with saline. In drug-treated cultures, including bleomycin-treated cultures, cells showing features of apoptosis were observed (fig 5.2 D). Morphological studies also revealed that 2-ME induced metaphase characterized by abnormal alignment of chromosomes (fig 5.2 F), possibly due to disruption of microtubules. Apoptotic cells were also observed in 2-ME treated cultures (fig 5.2 F). In a previous study conducted on the breast cancer cell line, MCF-7, 2-ME was shown to induce a metaphase block which ultimately lead to the induction of apoptosis. It is possible that in this study, the 2-ME induced abnormal alignment of chromosomes during metaphase prevented cells from proceeding beyond this phase of the cell division cycle, and resulted in BME cell morphology characteristic of apoptosis. Microscopic observations of cells treated with vinca alkaloids were inconclusive. While light microscopy provides useful information on morphological changes in cells undergoing apoptosis, chromatin condensation and DNA fragmentation have been cited as early and relatively unequivocal hallmarks of apoptosis. [6.,9,10]

It has also been suggested that as a rule, classification of cell death in a given model should always include morphological examination coupled to at least one other assay. [6] Therefore, in this study, DNA fragmentation and chromatin condensation were assessed to further determine possible apoptosis.

When DNA extracted from cells treated with bleomycin, mitomycin C, 2-ME, taxol, nocodazole and cytochalasin D was analyzed using gel electrophoresis, characteristic internucleosomal “ladders” of DNA fragments were found (fig 5.3 and 5.4). In respect to the intensity of DNA ladders, a dose response was apparent in samples treated with bleomycin and mitomycin C, with greater evidence of DNA fragmentation in cells exposed to higher drug doses (fig 5.3).

Internucleosomal DNA fragmentation is a biochemical indicator of apoptosis, and is attributable to the activation of endogenous endonucleases which cleave DNA. [9]

Apoptosis was also determined by AO labelling of BME cells treated with the various test drugs. Bleomycin increased acridine orange staining of BME cell nuclei (fig 5.5 B). Increased AO-staining of cell nuclei was also observed in cells treated with test drugs, indicating that these drugs induced apoptosis.

In this chapter it was demonstrated that test drugs induced BME cell apoptosis. Since a balance between endothelial cell proliferation and apoptosis ensures homeostasis of the vascular endothelial cell population, [1] and proangiogenic factors promote neovessel formation by inhibiting apoptosis, increased endothelial cell apoptosis may lead to angiogenesis inhibition. Indeed, several potent angiogenesis inhibitors, such as angiostatin and endostatin, were reported to inhibit angiogenesis in part by inducing endothelial cell apoptosis. [3] Furthermore, apoptosis has been shown to increase five-fold in haemangiomas undergoing regression. [13] Therefore the apoptotic effects of these test drugs on endothelial cells suggests that they may have potential in inhibiting the excessive angiogenesis associated with haemangioma development.

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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.

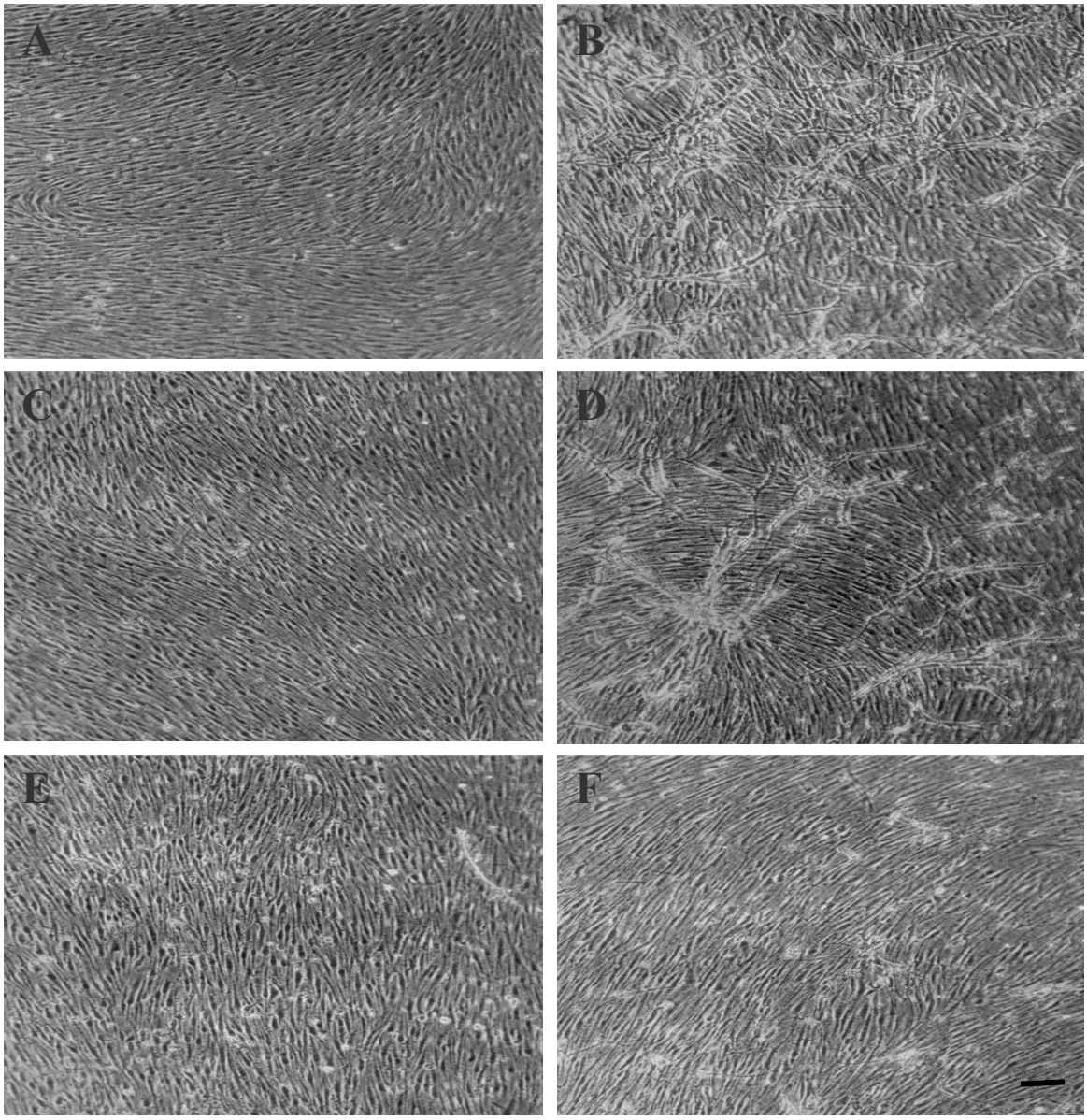


Figure 6.1. Phase contrast views of the effects of bleomycin on bovine microvascular endothelial (BME) cells grown on collagen gel. (A) – untreated cells; (B)- cells treated with a combination of 30 ng/ml VEGF + 10 ng/ml bFGF. Cells in (C) and (E) were treated with 30 µg/ml + 100 µg/ml bleomycin respectively;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

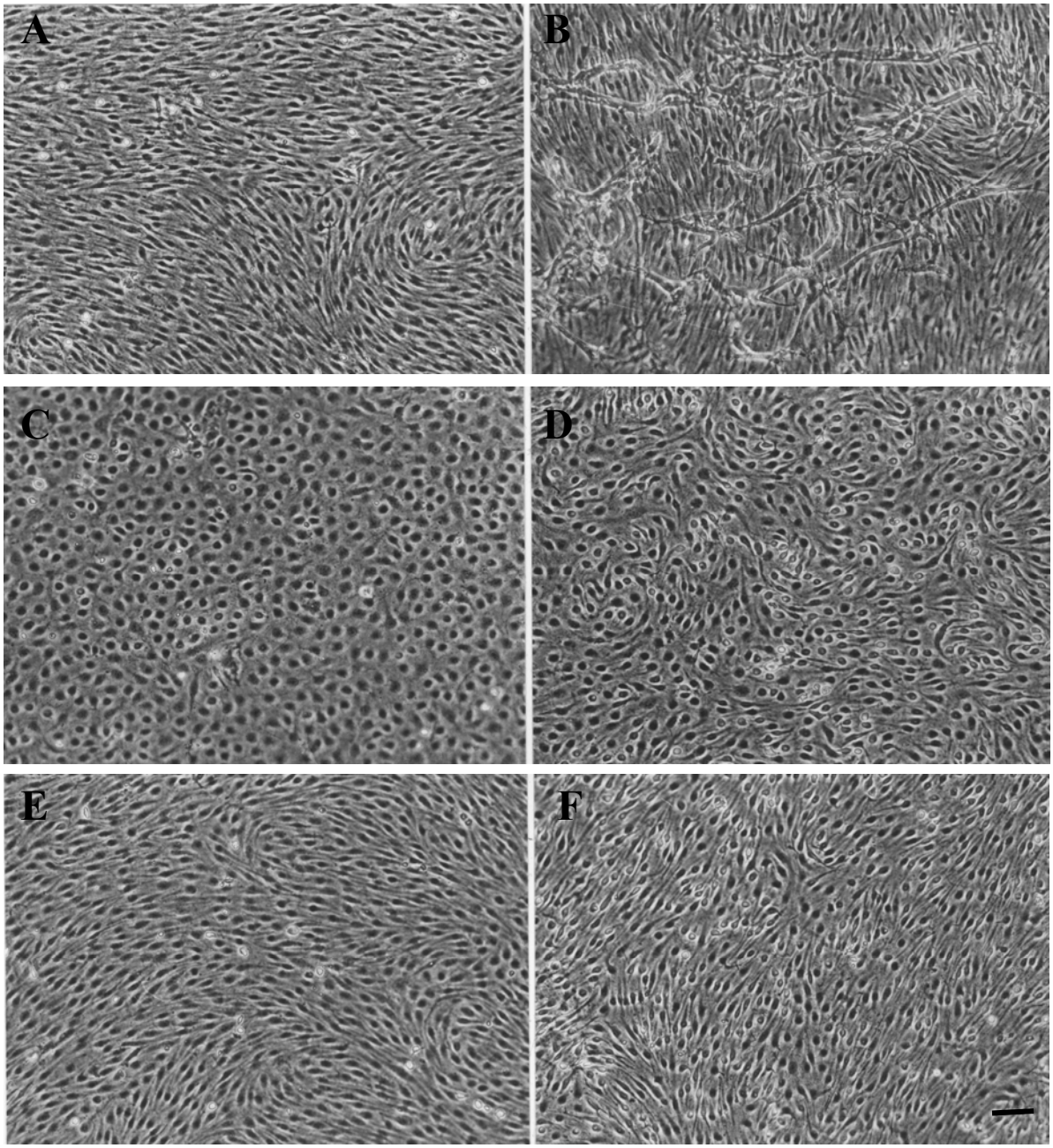


Figure 6.2. The effects of 2-methoxyestradiol (2-ME) and taxol on BME cells grown on collagen gel (Phase contrast). (A) Control untreated cultures; (B) bFGF-treated cultures; (C) 2-ME- and (E) Taxol-treated cultures; Cultures treated with a combination of bFGF and either (D) 2-ME or (F) Taxol. Bar =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

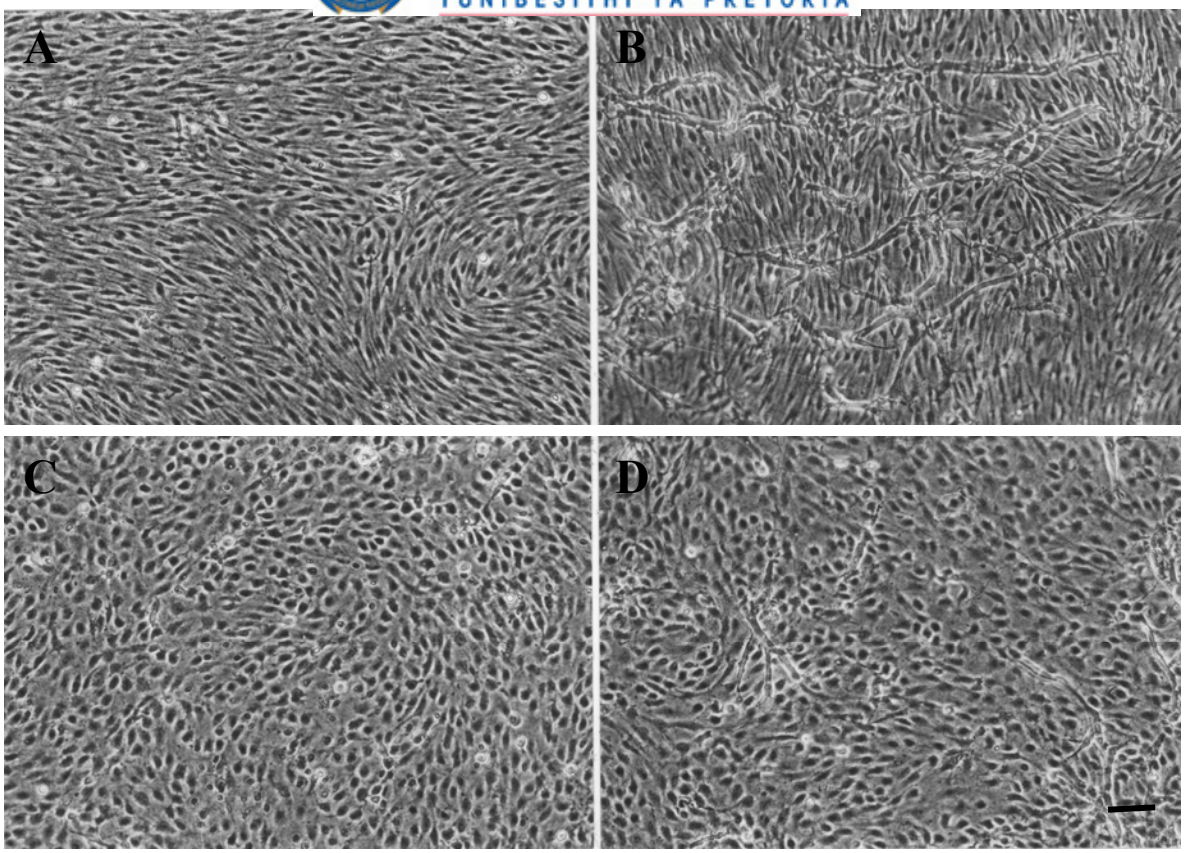
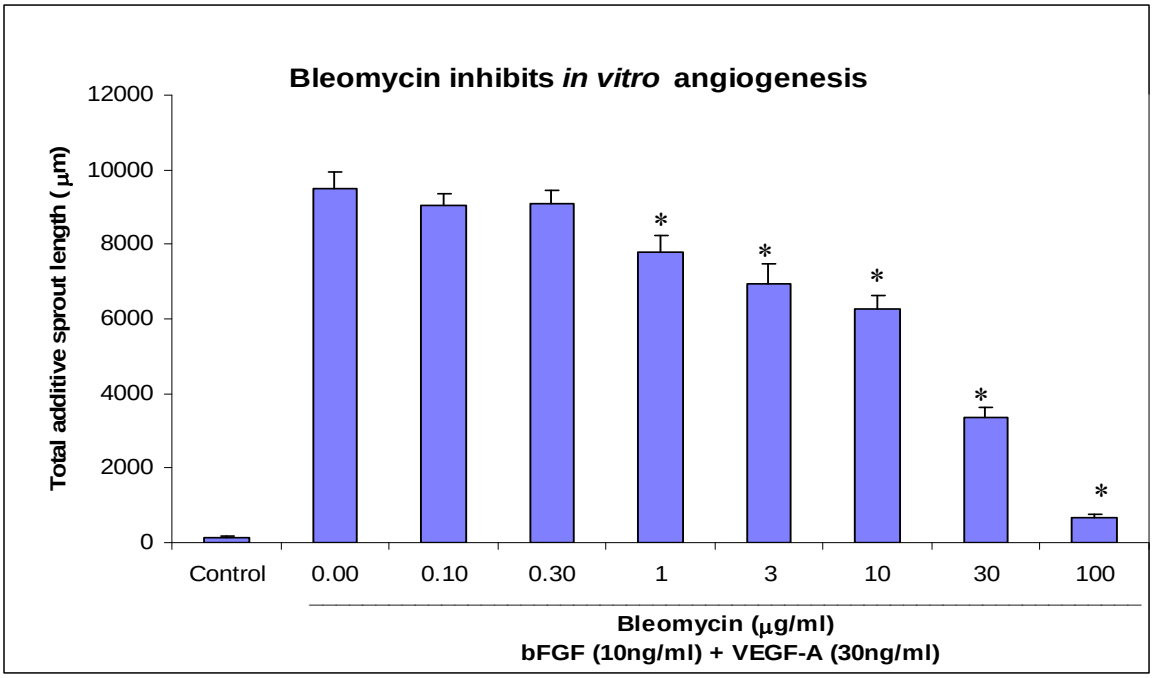


Figure 6.3. Effect of colchicine on BME cells grown on collagen gel (A and B are the same as in fig 6.2). (A) Control BME cells; (B) BME cells exposed to 10ng/ml bFGF; (C) Cultures exposed to colchicine only; (D) bFGF-treated cultures exposed to colchicine. Bar = 100 µm

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).



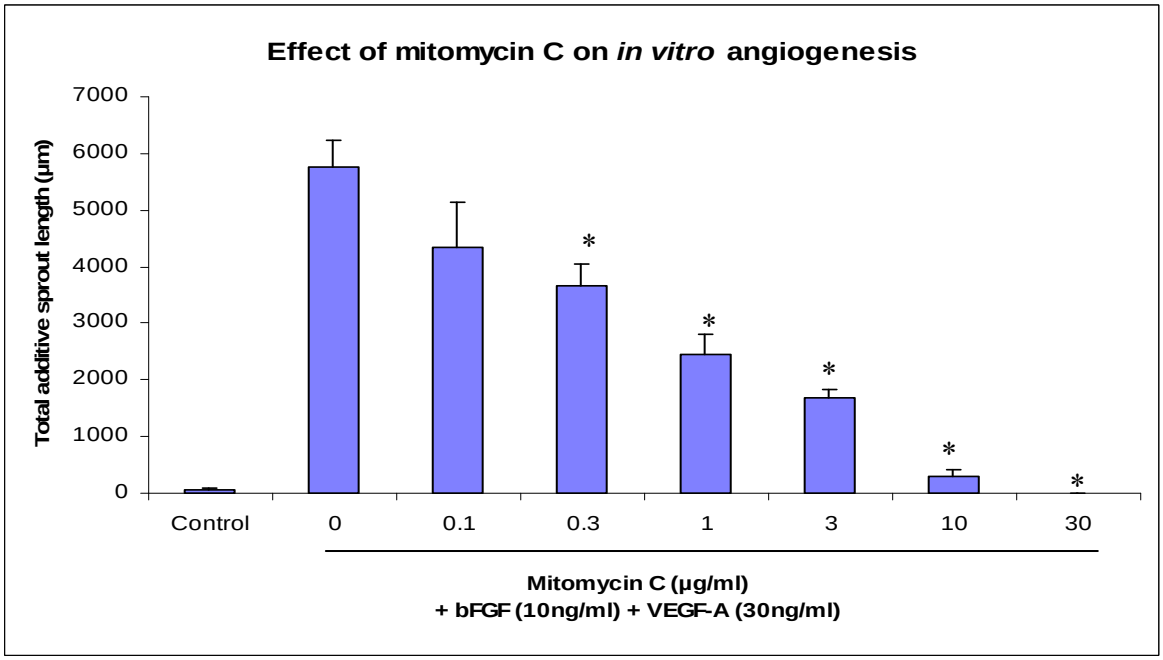
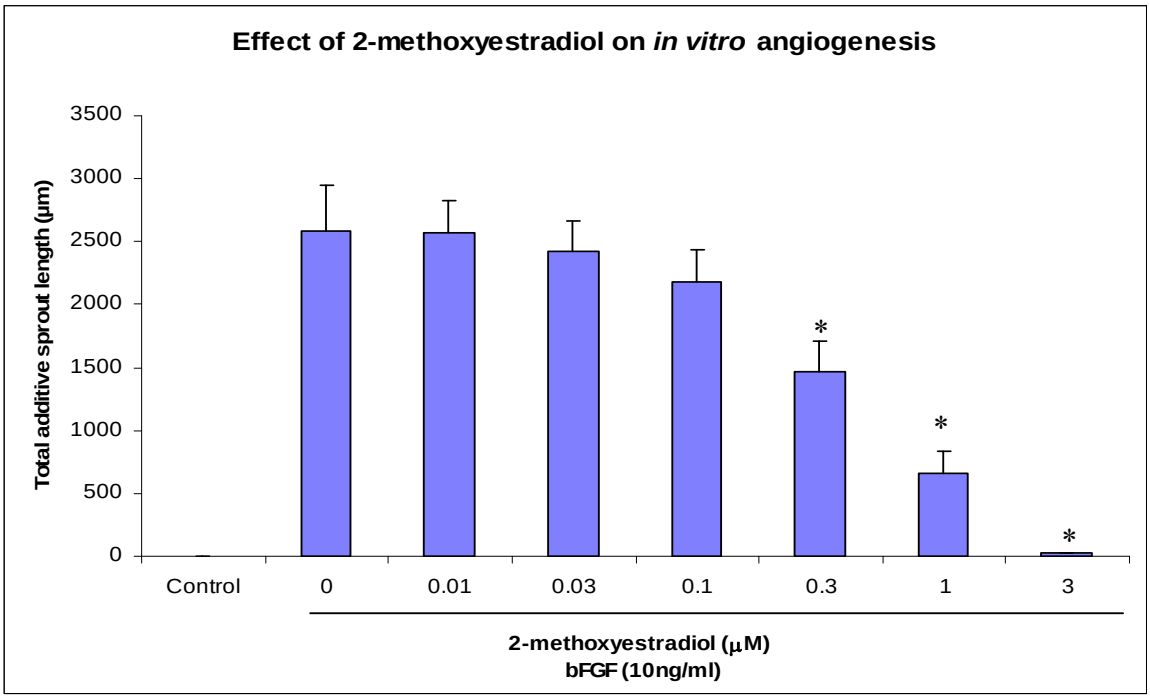


Figure 6.4. Quantitative analysis of the effects of bleomycin and mitomycin C on *in vitro* angiogenesis. Significant difference in the total additive sprout length between bFGF only -treated (0 µg/ml drug) and cells treated with a combination of bFGF + drug: * $P < 0.05$

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).



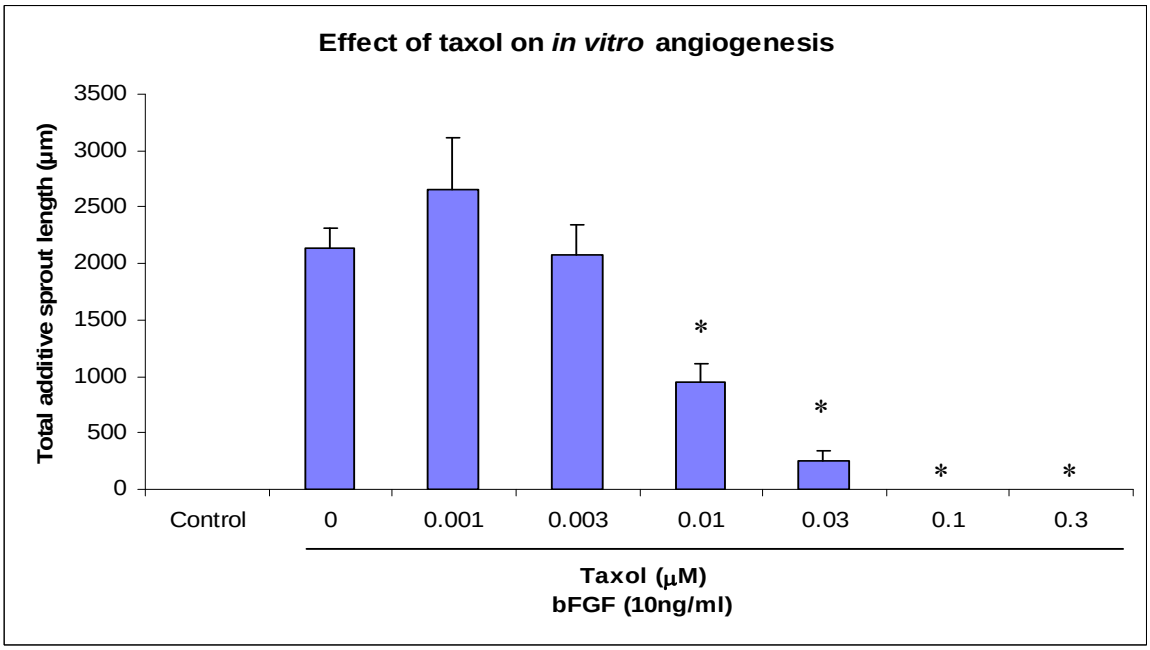
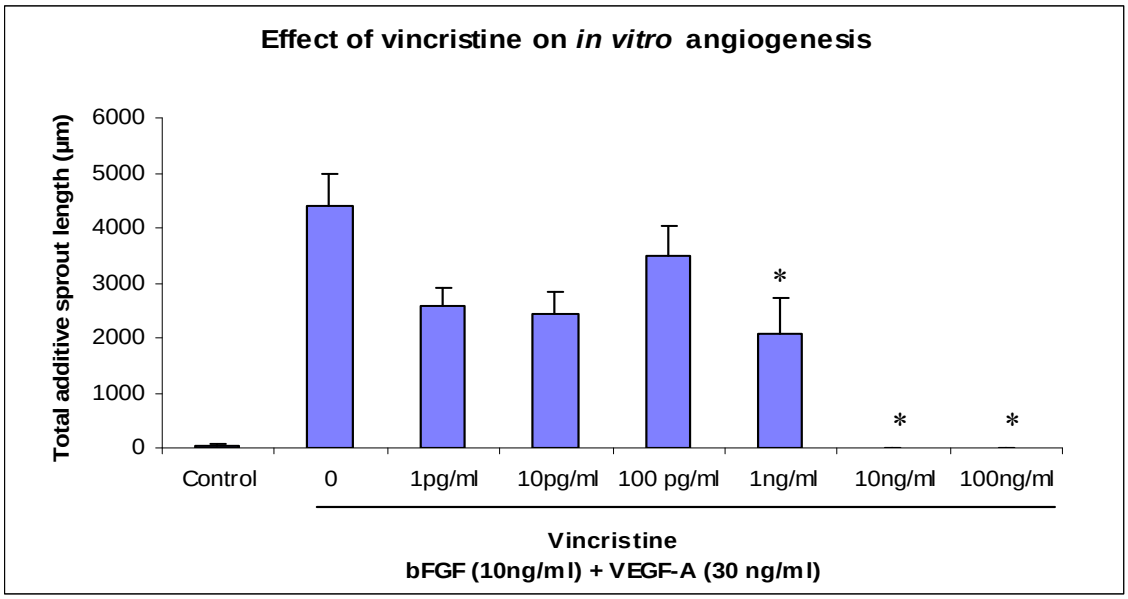


Figure 6.5. Quantitative analysis of the effects of 2-methoxyestradiol and taxol on *in vitro* angiogenesis. Significant difference in total additive sprout length between bFGF-treated (0 µM drug) and cells treated with a combination of bFGF and drug: * $P < 0.05$

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



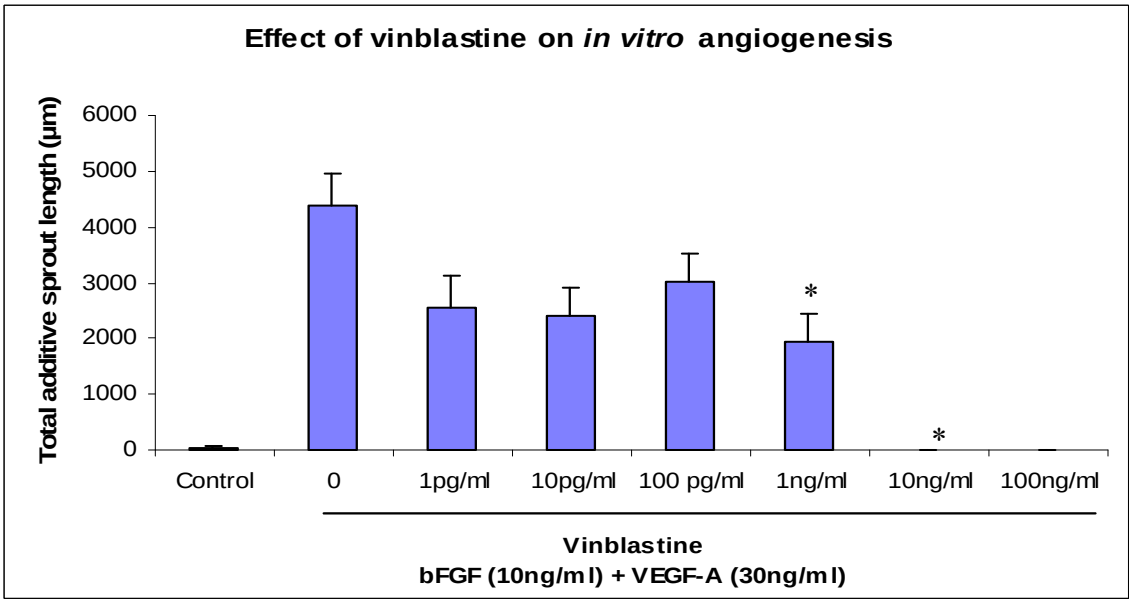


Figure 6.6. Quantitative analysis of the effects of vincristine and vinblastine on *in vitro* angiogenesis. Significant difference in total additive sprout length between bFGF only - treated (0 pg/ml drug) and cells treated with a combination of bFGF and drug: * $P < 0.05$.

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).

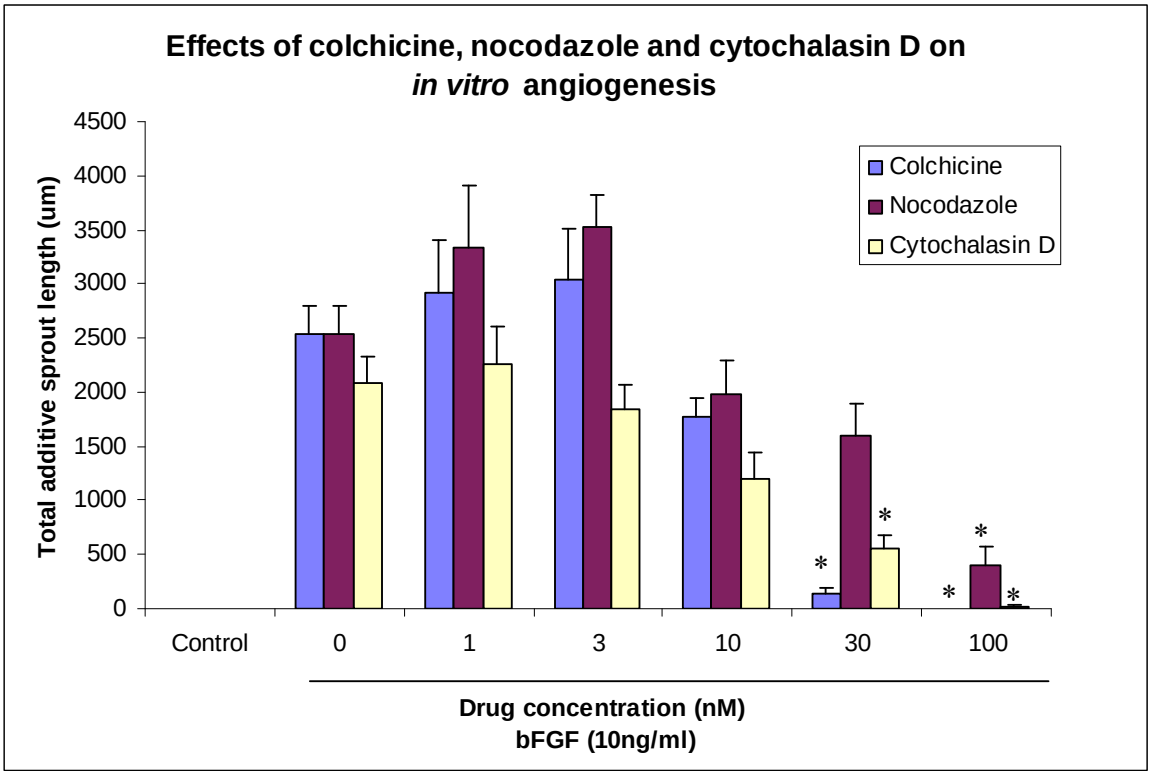


Figure 6.7. Quantitative analysis of the effects of colchicine, nocodazole and cytochalasin D on *in vitro* angiogenesis. Significant difference in total additive sprout length between bFGF only-treated (0 nM drug) and cells treated with a combination of bFGF and drug (colchicine, nocodazole or cytochalasin).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

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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CHAPTER 7

Effects of angiogenesis inhibitors on vascular tumour growth in an animal haemangioma model

7. 1. Introduction

In previous chapters, test drugs were shown to inhibit angiogenesis *in vitro* in assays designed to recapitulate each of the events that constitute the angiogenic process, namely, endothelial cell migration, endothelial cell growth, and the formation of capillary-like structures in a collagen matrix. However, preclinical investigation of these potential angiogenesis inhibitors requires final validation in an *in vivo* disease model. [1] One model cited as being useful for studying vascular tumours and tumour-associated angiogenesis, is based on the inoculation of mice with endothelial cells transformed by the polyoma middle T (Pym T) oncogene. [2,3]

The polyoma virus, a DNA tumour virus, induces neoplasms in a wide range of tissues in rodents, and has been used for many decades to study tumour development. [4] The middle T antigen of murine polyoma virus rapidly transforms endothelial cells, leading to the formation of vascular tumours in newborn mice. [4,5]

In vitro, endothelioma (End.) cells established from such tumours grow as immortalized cell lines. These End cells retain important features of endothelial cells, including expression of proteins characteristic of endothelial cells (vWF, CD31, MECA-32), and expression **of** vascular endothelial receptor, VEGFR-2. When cultured in fibrin gel in 3-D conditions, End. cells form cyst-like structures reminiscent of the histological structure of cavernous haemangiomas that develop in chimeric mice expressing mT oncogenes. [6] On the other hand, normal endothelial cells grown on a physiologically relevant 3-D substratum form capillary-like tube structures. [7,8] *In vivo*, in several different species, End. cells rapidly induce haemangiomas. [4] The ability of End. cells to induce vascular tumours in a murine model has not been reported for BME cells. However, in previous chapters, test drugs (bleomycin, mitomycin C, colchicine, vinblastine, 2-methoxyestradiol and taxol) were shown to inhibit various aspects of the angiogenesis process in BME cells.

Growth studies were thus undertaken using End. cells to determine whether these test drugs had any effect on this endothelial cell line prior to commencing with *in vivo* studies on a model that uses End. cells.

An initial objective of this study was to induce tumour growth in mice using endothelial cells transformed by the polyoma middle T antigen (Pym T). A further objective was to determine the effects of test drugs (bleomycin, mitomycin C, colchicine, vinblastine, 2-methoxyestradiol (2-ME) and taxol) on tumour growth in this murine haemangioma animal model. All mice were monitored for tumour development, and when tumours were present, tumour diameter was measured. Also, haematological analysis was undertaken in both control and drug-treated mice. To our knowledge, this is the first study that investigates both histological and haematological parameters in mice inoculated with End. cells. Also, the effects of these test drugs on a haemangioma model have not been reported before.

7. 2. Materials and Methods

7.2.1. Cell Maintenance

The endothelioma cell line, sEnd.2, derived from Pym T induced vascular tumours in the skin of the thorax of C57BL6 x 129 mice, was grown in Dulbeco's Modified Eagle's medium (Sigma, St Louis, MO, USA) supplemented with 10% foetal calf serum (Highveld Biologicals, Sandringham, SA), 10 nM glutamine, and 1% penicillin-streptomycin (Highveld Biologicals, Sandringham, SA). The cell line was maintained as monolayer cultures in a 37 ^{o}C incubator in a humidified atmosphere containing 5% CO_2.

Viable cells from stock flasks were determined using trypan blue exclusion prior to seeding procedures: cells from stock flasks were trypsinised and the cell suspension stained 1:1 with 0.2% trypan blue in phosphate buffered saline (PBS). Unstained and thus viable cells were counted with a haemocytometer.

7.2.2. Cell Growth

Cell growth studies were performed to assess the effect of bleomycin, mitomycin C, colchicine, vinblastine, 2-methoxyestradiol (2-ME) and taxol, on sEnd.2cell growth using crystal violet nuclear staining method as described by Gillies *et al*. (1983). [9]

Endothelioma cells were seeded into 24-well culture plates at a density of 10 000 cells per well and exposed to varying concentrations of test drugs for 48 hours. Control cells were exposed to vehicles (saline/ distilled water/ DMSO). At termination, cells were fixed with 1% glutaraldehyde in PBS for 15 minutes. Cells were then stained with a 1% solution of crystal violet (in H_2O) for 30 minutes. The chromophore was extracted with 500 µl of 0.1% Triton X-100 per well. The absorbance of samples was read at 570 nm on an ELx 800 Universal Microplate Reader. Three wells were analysed for each concentration. Cell growth studies were repeated three times for each drug.

7.2.3. Experimental Animals

Female C57BL6 mice (8 weeks old) were housed individually in a temperature-controlled room (21°C) with standard lighting conditions (12 L-12 D) at the University of Pretoria Biomedical Research Centre (UPBRC). The mice were given standard commercial food (EPOL) and water *ad libitum*. The administration of all drugs was performed with the assistance of a laboratory animal technologist. At the end of the study, the mice were terminated by cervical dislocation. Animal experiments were approved by the University of Pretoria Animal Use and Care Committee (project 16/2005).

7.2.4. Effects of Drugs on tumour development

Tumours were induced based on a method previously described by Sabapathy *et al.* (1997). [10] Mice were first injected with drugs as outlined in the schematic diagram of the study protocol (fig 7.1). Mouse endothelioma (sEnd.2) cells were then injected subcutaneously into the necks of the mice at a density of 2 X 10^6. In preliminary studies on group-housed mice, cells were injected into the flanks of the animals, however, due to scratching; the cells were injected into the necks of the animals in subsequent experiments. The mice were monitored every second day for tumour formation. The times taken for the onset of tumours and the latency period to form were recorded. Tumour size was measured with a micrometer. The incidence of tumour development was determined by calculating the percentage of mice that developed tumours in each treatment group.

At termination, tissue was dissected and fixed in 4% paraformaldehyde in PBS. Samples were then embedded in paraffin, 5 µm sections were cut and subsequently stained in eosin and hematoxylin (tissue samples were processed at the Department of Pathology, Faculty of Veterinary Sciences, University of Pretoria). Blood samples were collected in heparinised tubes for haematological analysis. The red cell count, white cell count, platelet numbers, hematocrit and haemoglobin levels were measured using a hemocounter, Cell-Dyne 3700 (Abbott, IL, USA). Haematological analysis was undertaken at the department of Clinical Pathology, Faculty of Veterinary Sciences, University of Pretoria. A schematic representation of the experimental design is outlined in Fig 7.1.

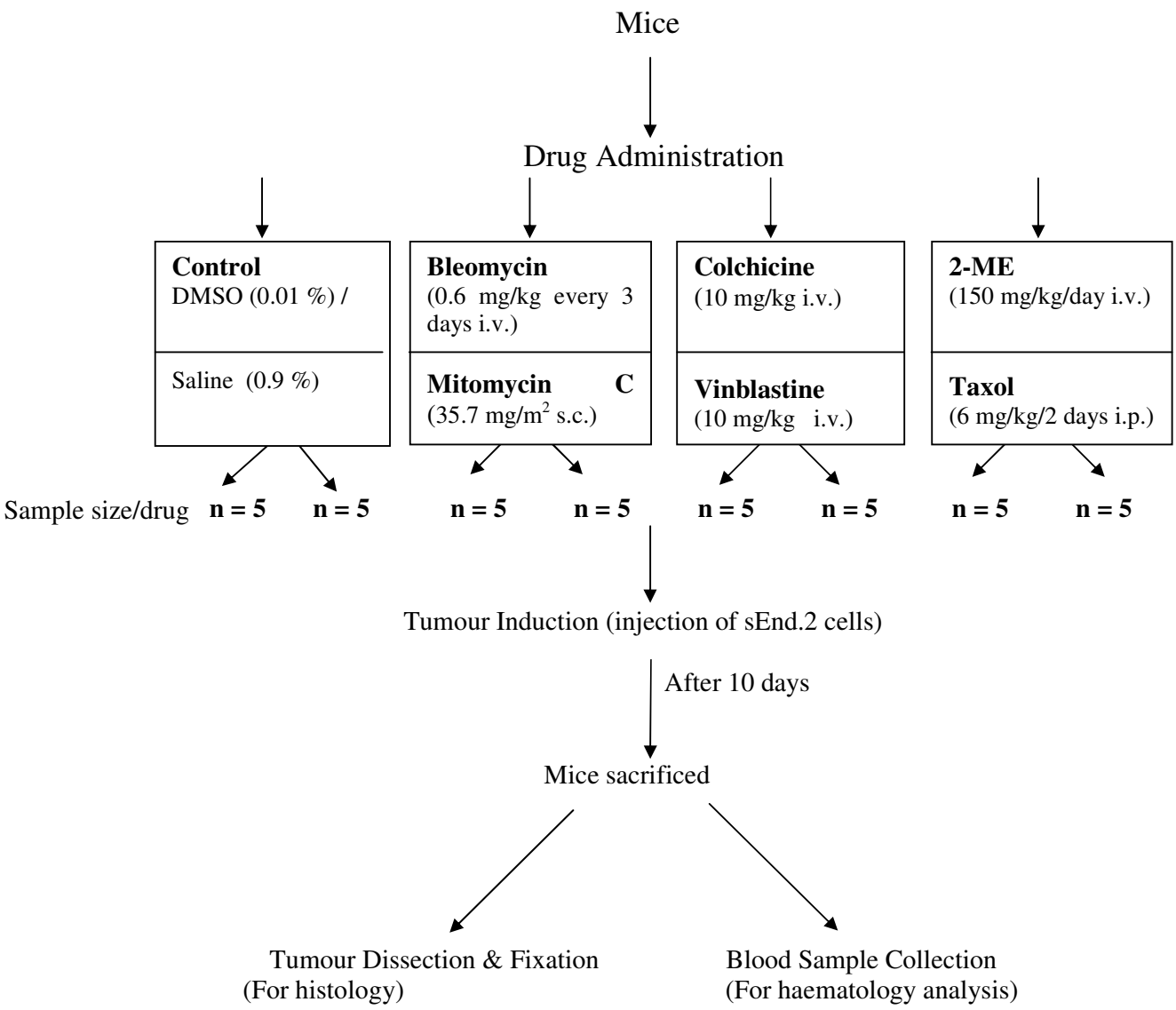

Figure 7.1. Schematic diagram of the experimental design.

Table 7.1. Drug doses used in this study. Current dose refers to drug doses employed in the present study; previous study refers to drug doses from previous reports by different authors (references are supplied under ref).

Drug	**Current Dose**	**Previous Study**	**Ref**
*Bleomycin	0.6 mg/kg every 3 days i.v.	Antitumour effect	Gaeng *et al*., 1995 [11]
Mitomycin C	35.7 mg/m^2 s.c.	Toxicity of anticancer agents	Freirech *et al.,* 1966 [12]
*Colchicine	10 mg/kg i.v.	Antitumour effect	Nihehi, 1999 [13]
Vinblastine	10 mg/kg i.v.	Antitumour effect	Nihehi, 1999 [13]
2-ME	150 mg/kg/day i.v.	Angiogenesis and breast cancer inhibition	Klauber *et al*., 1997 [14]
Taxol	6 mg/kg every 2 day i.p.	Angiogenesis and breast cancer inhibition	Klauber *et al*., 1997 [14]

The doses of various drugs employed in this study were based on previous studies (references provided in table) and on preliminary studies undertaken in mice.
* Dose modified after initial studies on 80 mice.

7. 3. Data Analysis

Statistics were performed using Stata Release 8. The concentration effect of different drugs on sEnd.2 cell growth was analyzed by comparing control and drug-treated cells. One-way ANOVA was used, and where significance was reached, Bonferroni's method was used. The limit of statistical significance was defined as $P<0.05$.

To analyze data from animal studies, tumour diameter in control and drug treated mice was compared using the two-tailed Mann-Whitney test. Significance was designated at $P<0.05$. The unpaired t-test was performed to determine if there was a difference in blood parameter values (hematocrit, haemoglobin concentration, red cell count, white cell count and thrombocyte/ platelet count) between control and drug-treated mice. The analysis was repeated using the Welch test, which does not assume equal variances, and the non-parametric Mann-Whitney test. Again, significance was designated at $P<0.05$.

7. 4. Results

7.4.1. Cell Growth

The effects of test drugs on sEnd.2 cell growth were determined using the crystal violet nuclear staining assay. Bleomycin and mitomycin C induced a reduction in the percentage of viable cells (Fig 5.2). Significant inhibition of cell growth by bleomycin and mitomycin C was observed following exposure to doses of 10 and 1 µg/ml respectively. The IC_{50} values for the drugs are listed in table 7.2.

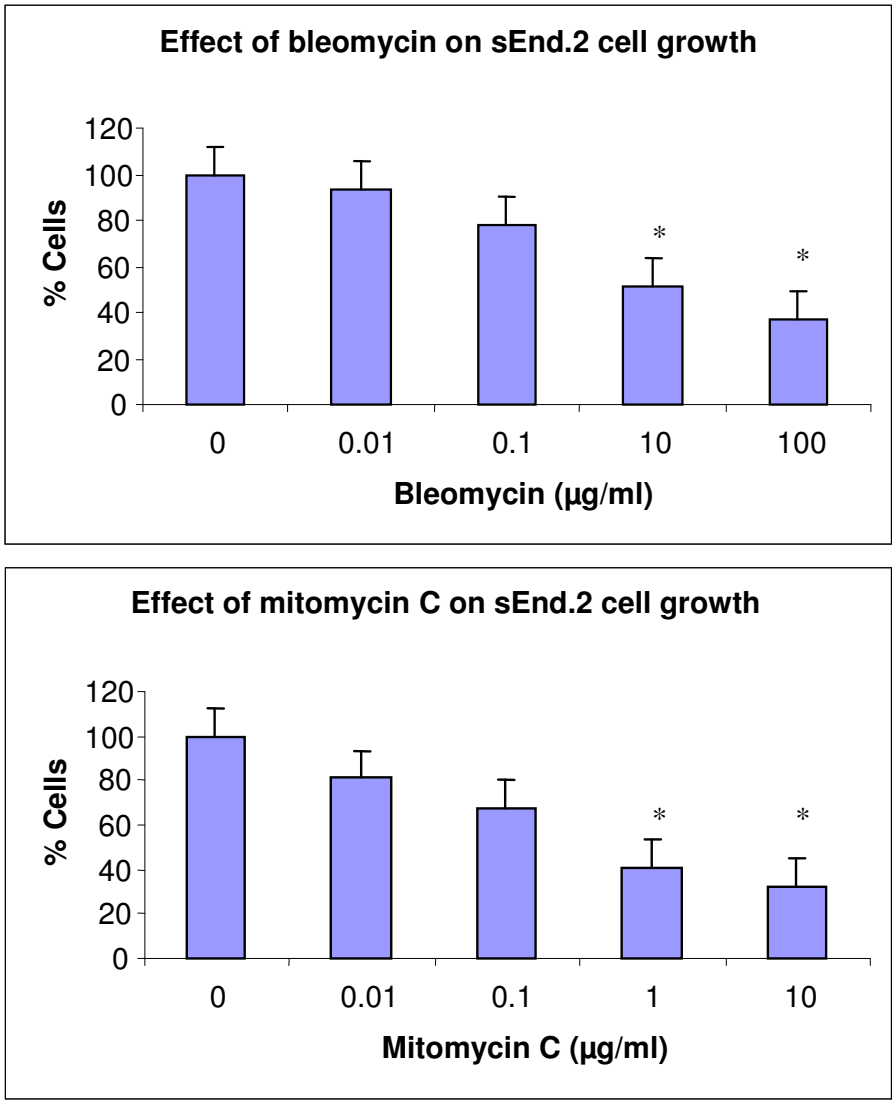

Figure 7.2. Effects of bleomycin and mitomycin C on sEnd.2 cell growth.
* Significant difference between the percentage of viable cells in control and drug-treated cultures ($P<0.05$).

2-Methoxyestradiol and taxol decreased the percentage of cells at doses of 0.1 µM. The IC_{50} of 2-ME for sEnd.2 cells was 0.016 while that of taxol was 0.1 µM (table 7.2). Increasing the dose of either 2 ME or taxol from 1 to 10 µM did not induce a further reduction in cell numbers.

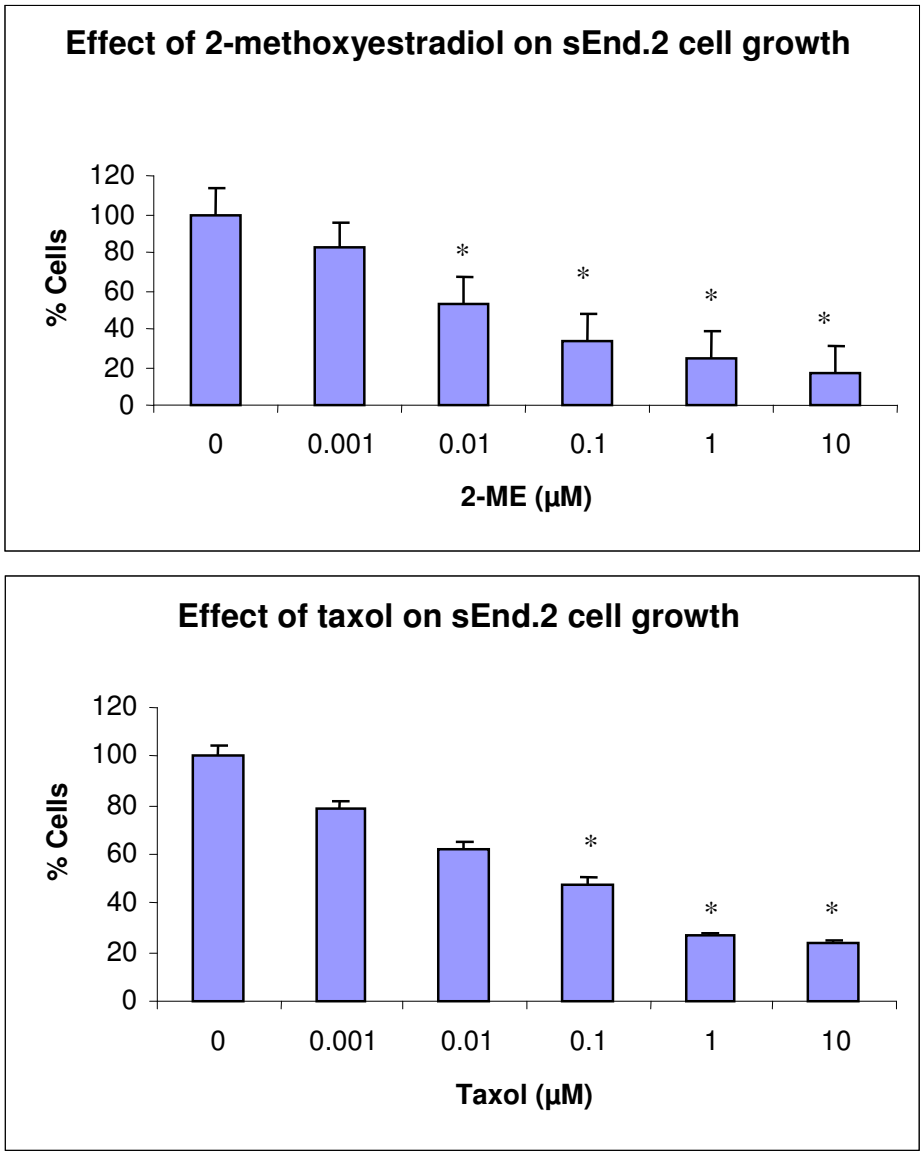

Figure 7.3. Effects of 2-Methoxyestradiol and taxol on sEnd. 3 cell growth.
* Significant difference between the percentage of viable cells in control and drug-treated cultures ($P<0.05$).

Colchicine induced a reduction in the percentage of viable cells at a dose of 0.001 µM. The IC_{50} of this drug in sEnd.2 cells was 0.05 µM. Significant inhibition of growth in vinblastine treated cultures was observed at doses of 0.1 ng/ml.

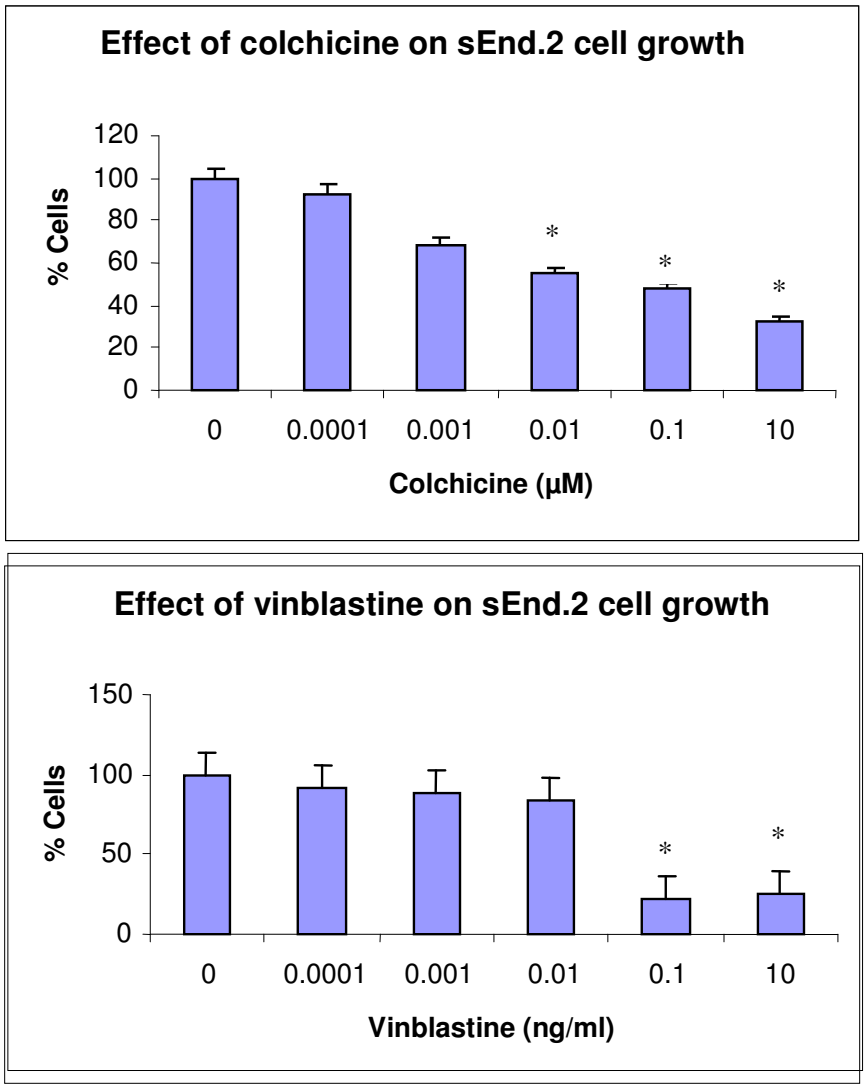

Figure 7.4. Effects of colchicine and vinblastine on sEnd.2 cell growth.
* Significant difference between the percentage of viable cells in control and drug-treated cultures ($P<0.05$).

Table 7.2. Concentration values of test drugs required to inhibit sEnd.2 cell growth by 50% (IC_{50} values).

Drug	**Cell Type**	**IC_{50}**
Bleomycin	sEnd.2 cells	2.108 µM (3.162 µg/ml)
Mitomycin C	sEnd.2 cells	1.683 µM (0.562 µg/ml)
2-Methoxyestradiol	sEnd.2 cells	0.016 µM
Taxol	sEnd.2 cells	0.100 µM
Colchicine	sEnd.2 cells	0.050 µM
Vinblastine	sEnd.2 cells	0.044 µM (0.039 ng/ml)

7.4.2. Tumour Development

In the control group, a tumour mass formed in each mouse at the site of injection 5-7 days after inoculation with sEnd.2 cells. The observation period was up to 10 days following injection of the mice with the cells. Tumour diameter was decreased in mice treated with varying doses of taxol (Fig 7.5). Also, the percentage of mice developing tumours was decreased from 100% (control) to 60% in mice treated with 1.2 mg/kg taxol. Tumour incidence was further decreased to 40% in mice treated with doses of 3 mg/kg of the drug. At doses of 6 mg/kg taxol, no tumours were observed.

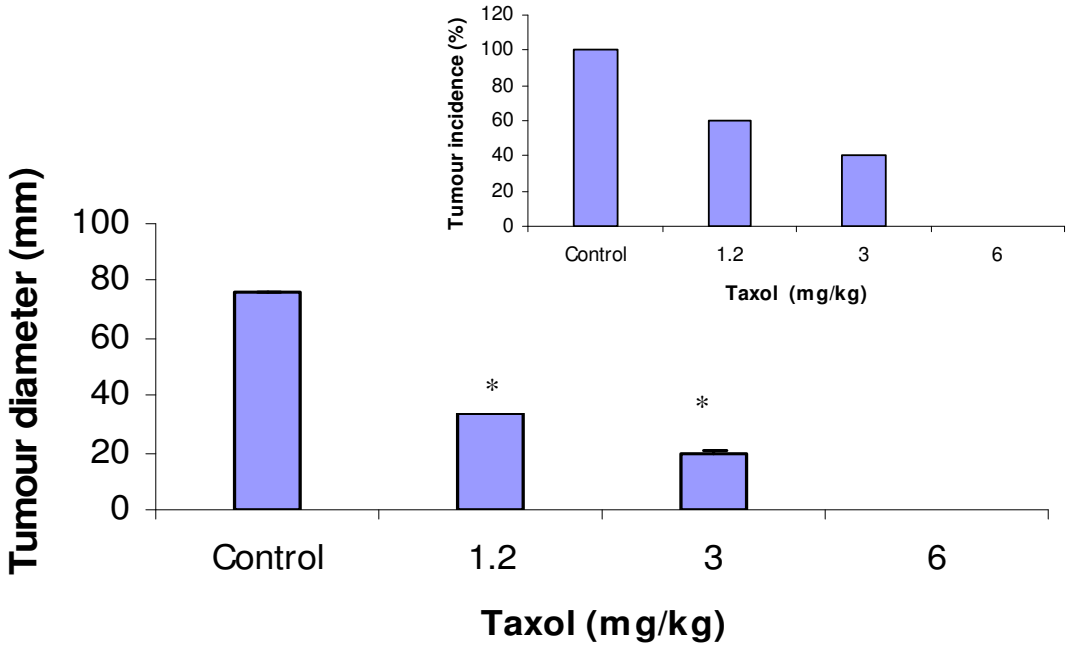

Figure 7.5. Effects of varying doses of taxol on tumour growth in mice inoculated with sEnd. 2 cells. (n = 5; 6mg/kg n = 3); * $P < 0.05$.

This study was repeated using bleomycin, mitomycin C, colchicine, vinblastine, 2-ME and taxol. These test drugs inhibited tumour development with varying potency (Fig 7.6). Tumours observed in bleomycin-, mitomycin C- and colchicine-treated mice were smaller in diameter compared to control mice. Tumours developed in all mice treated with bleomycin. However, the incidence of tumour development was reduced in mitomycin C- and colchicine-treated mice. No tumours were observed in mice treated with vinblastine, 2-ME or taxol.

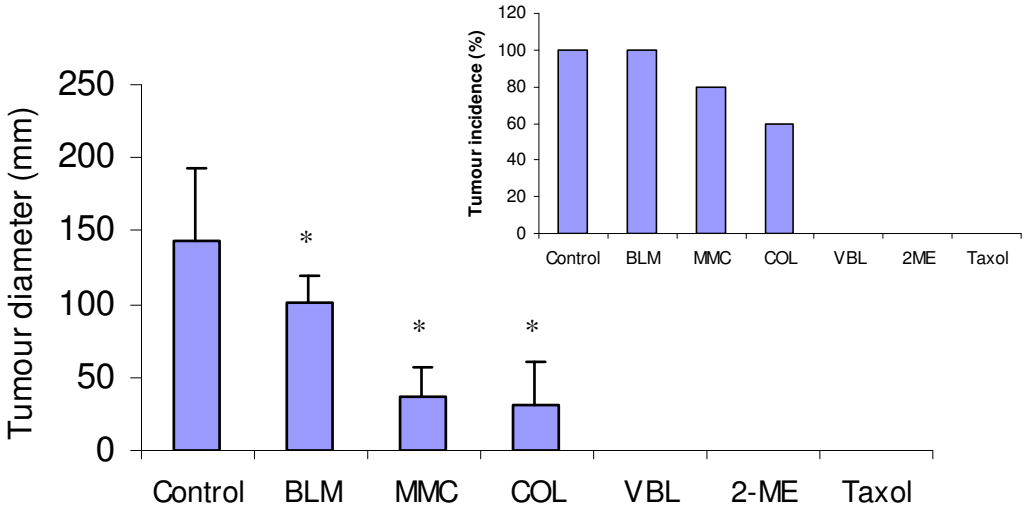

Figure 7.6. Effects of test drugs on tumour growth in mice inoculated with sEnd. 2 cells. n = 5, except for taxol. (n = 3); * $P < 0.05$.

BLM = Bleomycin; MMC = mitomycin C; COL = colchicine; VBL = vinblastine;
2-ME = 2-methoxyestradiol

7.4.3. Histology

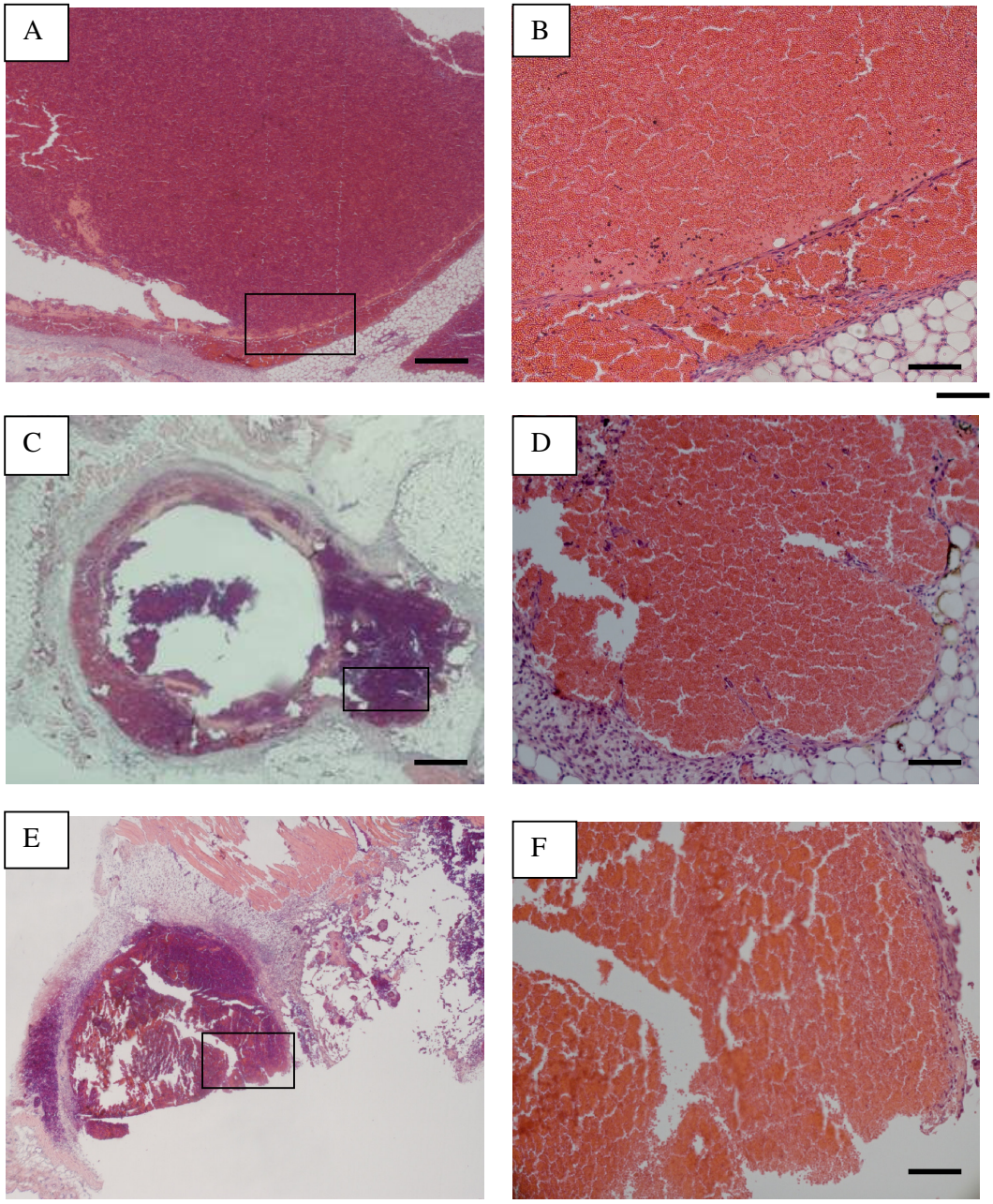

Figure 7.7. Histology of sEnd.2 cell-induced vascular tumours in mice. A and B: histology of a tissue sample from a control mouse showing with a vascular tumour composed of a layer of endothelial cells surrounding a large blood-filled lumen and inflammatory cells (stained purple). C and D: a vascular tumour in a mouse treated with bleomycin; E and F: a vascular tumour in a mouse treated with colchicine.

In control mice, a layer of endothelial cells lining a blood filled cavity was observed. Inflammatory cells were also observed within and around the cavity. In bleomycin-, mitomycin C, and colchicine-treated mice, the endothelial cell lining was not observed (immonuhistochemistry will be undertaken to confirm this observation). The blood-filled cavity was reduced in size, however, inflammatory cells were observed in bleomycin- and mitomycin C-treated mice.

7.4.4. Haematology

The red cell count (RCC) in control tumour bearing mice was markedly decreased. Haemoglobin concentration and hematocrit percentage were also decreased in tumour bearing control mice. There was no significant change in the white cell count of mice inoculated with sEnd.2 cells when compared to mice injected with saline only. However, platelet numbers were decreased severely in tumour bearing control mice. Mice were treated with varying doses of taxol, which has previously been adopted in various studies on tumour angiogenesis in mice and was recently included (in combination with other drugs) in clinical trials of tumour angiogenesis. [15]

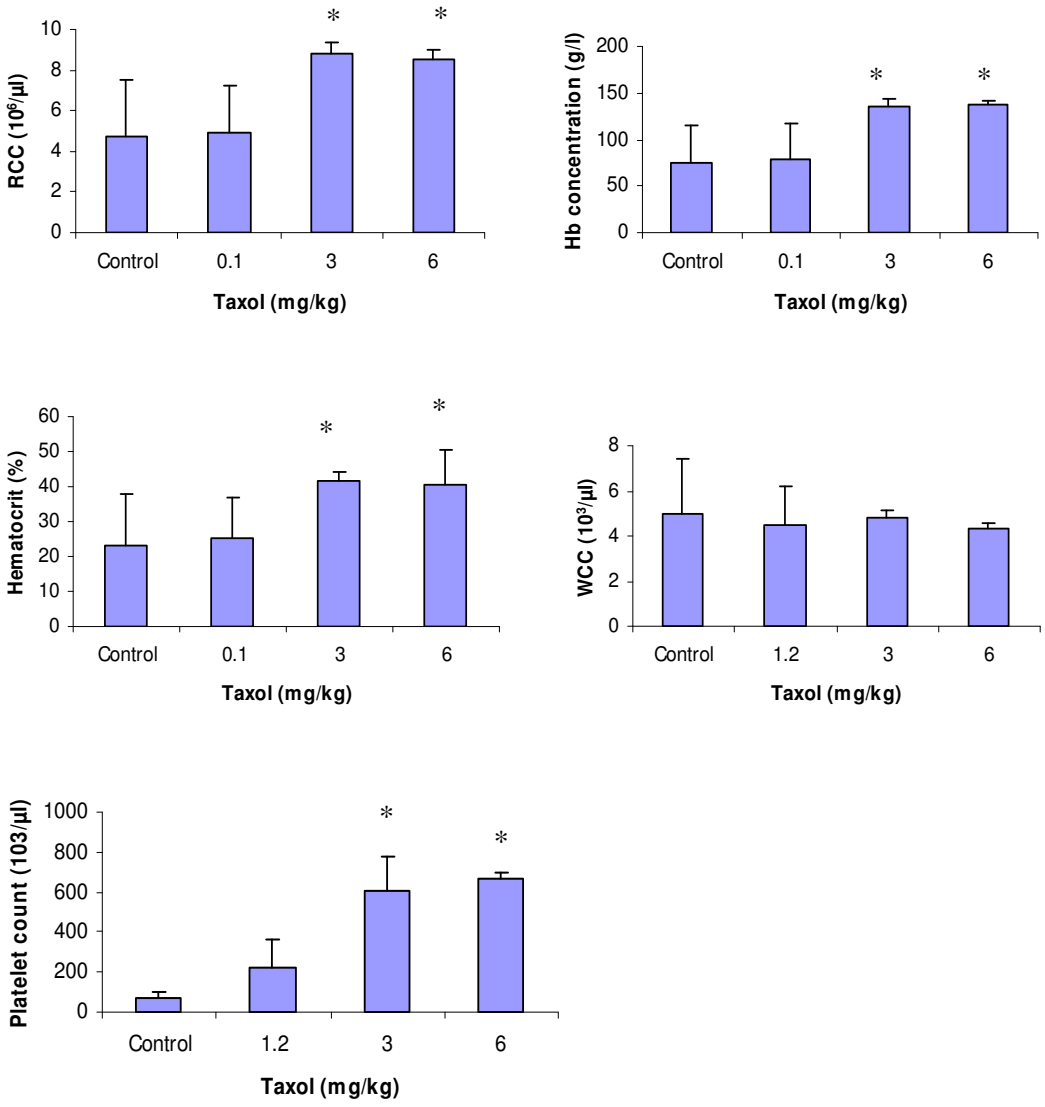

Figure 7.8. Blood parameter values (mean ± SEM) in control and taxol-treated mice. n = 5; * $P < 0.05$. RCC = red cell count; Hb = haemoglobin; WCC = white cell count.

At lower taxol doses (0.1 mg/kg), the red cell count appeared to be unaffected. Higher doses of taxol (3 and 6 mg/kg) lead to significant increases in the red cell count ($P < 0.05$). Taxol had no effect on either haemoglobin concentration or hematocrit percentage at 0.1 mg/kg, however, the two parameters increased significantly in mice treated with 3 and 6 mg/kg of taxol. Taxol had no observable effect on white cell numbers at doses used in this study. However, platelet numbers showed a significant increase only in mice treated with 3 and 6 mg/kg.

Results of the repeat study showed that the red cell count (RCC) was decreased in control tumour bearing mice (fig 6.8). The decrease in RCC correlated with a decrease in haemoglobin concentration. The mean hematocrit percentage was reduced significantly in mice injected with sEnd.2 cells compared to mice injected with saline only. There was no significant change in the levels of white blood cells following injection of mice with sEnd.2 cells (results not shown). Platelet numbers were severely reduced in tumour bearing control mice.

Following treatment of mice with bleomycin the RCC was increased in these animals. A more pronounced increase in red cell count was observed in mice treated with mitomycin C and the cytoskeletal-disrupting agents. The increase in RCC correlated with an increase in haemoglobin levels in drug treated mice. Hematocrit was also increased in sEnd.2 cell injected mice treated with bleomycin, mitomycin C, and colchicine. However, a more pronounced increase in hematocrit was observed in mice treated with vinblastine, 2-ME, and taxol.

There was a severe decrease in the number of platelets following injection of mice with sEnd.2 cells control mice, and the mean platelet count averaged 300 x 10^3 µl/ml. The platelet count was raised in bleomycin and mitomycin C treated mice. Platelet numbers were also increased in mice treated with cytoskeletal-disrupting drugs.

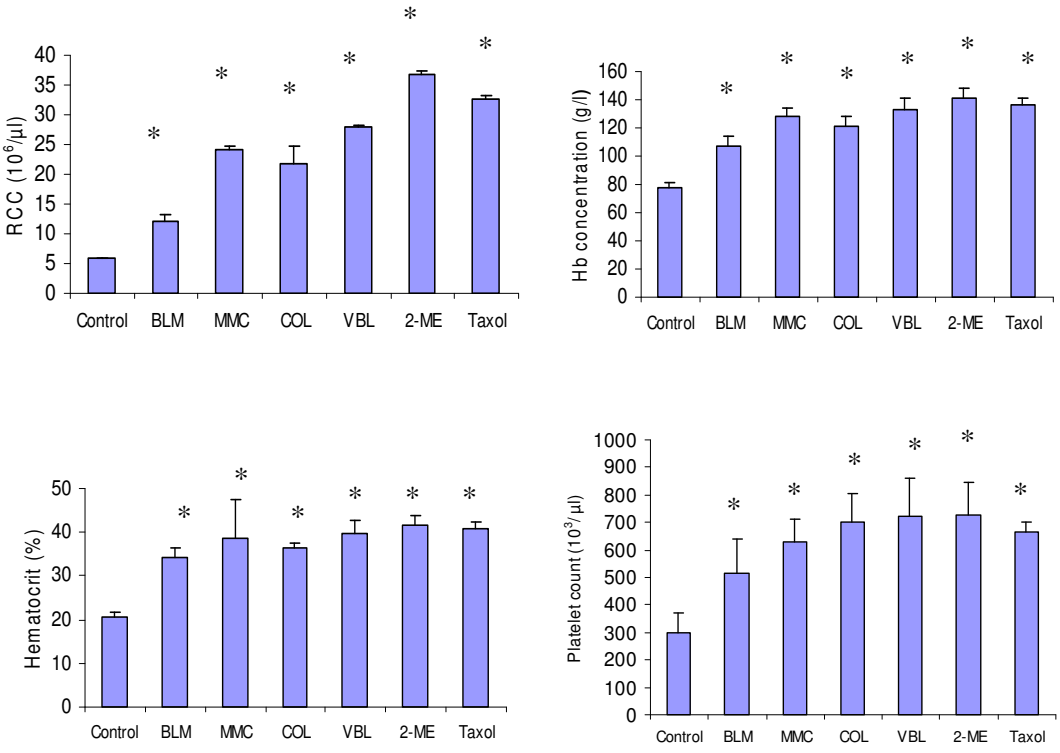

Figure 7.9. Blood parameter values (mean ± SEM) in control and drug-treated mice. n = 5, except for taxol (n = 3); * $P < 0.05$. RCC = red cell count; Hb = haemoglobin.

BLM = Bleomycin; MMC = mitomycin C; COL = colchicine; VBL = vinblastine; 2-ME = 2-methoxyestradiol

7. 5. Discussion

Vascular tumours developed in the subcutaneous tissue in 100% of the control mice injected with sEnd.2 cells. These tumours were partially encapsulated and were composed of soft dark tissue which exuded blood upon sectioning. According to Williams *et al.* (1989) End. cells expressing the middle-T antigen are the primary cause of pym T-induced tumours, since non-proliferating mitomycin C-treated End. cells were able to induce such tumours. [15]Also, the continuous presence of End. cells has been cited as being necessary to maintain the haemangioma. [15] On the other hand, studies have shown that injection of primary non-transformed endothelial cells induced no observable lesions. [15]

Histological sections from control mice showed tumours of variable size with blood filled cavities. [16] These vascular tumours, referred to in literature as haemangiomas, were associated with haemorrhage. Previous studies showed that lesions were formed by recruitment and migration of host endothelial cells to the site of injection. [16-18] According to Taraboletti *et al.* (1993), Pym T oncogene transformed endothelial cells produce a factor that stimulates and directs the migration of normal host-derived endothelial cells; this in turn contributes to haemangioma formation *in vivo*. [17] Indeed, both injected End. cells and recruited normal host endothelial cells have been implicated in the pathogenesis of mouse PymT-induced vascular tumours, although the degree to which either of these cells contribute to tumour formation remains to be elucidated.

With regard to the morphology of End. cell-derived vascular tumours, Pepper (1997) pointed out a number of significant morphological differences between infantile haemangiomas and these tumours: [19] i) previously histology sections of PymT induced vascular tumours revealed very little or no mitotic activity while juvenile haemangiomas are characterised by rapidly proliferating endothelial cells, ii) PymT induced tumours are characterised by the presence of host-derived endothelial cells; there appears to be no evidence of host-cell recruitment in the pathogenesis of juvenile haemangiomas. [19]

Results from this study further revealed that the haematological features of the pymT-induced tumours were as follows: red blood cells were decreased in tumour bearing control mice; the haemoglobin levels also decreased severely and correlated with the decrease in RBC.

The mean hematocrit value in control tumour bearing mice reduced significantly compared to mice injected with saline only (results not shown). Tumour mice also developed severe thrombocytopenia, with a mean platelet count of ~ 300 x 10^3 µl/ml, while there was no significant change in the white cell count. These observations were consistent with the diagnosis of haemangioma associated with the Kasabach-Merritt syndrome (KMS) in human beings, which is characterised by thrombocytopenia and microangiopathic anaemia. [20] However, in human beings changes in the white cell count have not been associated with these vascular lesions.

Vascular tumours associated with KMS, although closely related to infantile haemangioma based on biologic behaviour, were found to be histologically different from IH, and have been termed kaposiform haemangioendothelioma (KHE). [21-23] Previously, ultrastructural observation of KMS-associted tumours revealed 'widened intercellular junctions and poorly formed basement membranes'. Sarkar *et al.* (1997) attributed the exodus of platelets and plasma into the lesions to these histological observations. [23]

Effects of antitumour agents (bleomycin, mitomycin C, colchicine, vinblastine, 2-methoxyestradiol and taxol) on tumour growth in this haemangioma animal model were assessed. The tumour size was decreased in bleomycin-, mitomycin C-, and colchicine treated mice. Other cytoskeletal–disrupting agents, vinblastine, 2-ME and taxol completely inhibited tumour development. The histological analysis of tumours in drug-treated mice revealed discrete blood filled cavities. In mitomycin C-treated mice, these cavities were lined by a discontinuous layer of endothelial cells. In other mice, this layer of endothelial cells was not observed. Also, fewer capillary vessels and a reduced inflammatory response were observed in histological sections of drug-treated mice when compared to control mice.

The inhibition of tumour growth by test drugs correlated with a recovery in the red cell count. Also, the haemoglobin concentration, hematocrit percentage, and platelet count increased significantly ($P<0.05$) in mice treated with test drugs. According to Enjolras *et al.* (1997), thrombocytopenia in patients with vascular lesions associated with KMS is 'constant and severe', and the presence of platelets inside the lesion appears to sustain the growth of the cellular component of the tumour. [20]

Thus according to Enjolras *et al.* (1997) the aim of therapeutic intervention should be 'to increase the platelet count to a minimum acceptable level'. [20] Accordingly, the test drugs used in this study stimulated the recovery of platelet numbers, especially cytoskeletal-disrupting agents, implying that these drugs may have potential in treating vascular tumours complicated by the Kasabach-Merritt syndrome.

In conclusion, this study demonstrated that test drugs inhibit haemangioma development *in vivo* in a mouse model, and induced recovery of affected haematological parameters. These observations, coupled with the antiangiogenic effects of these drugs observed in previous chapters, indicate that these drugs may have potential in the treatment of haemangiomas of infancy.

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CHAPTER 8

Conclusion

To determine spill-over levels of bleomycin following intralesional therapy, a method for the assay of the drug in plasma was developed. To investigate bleomycin's mode of action in inhibiting haemangioma development, the effects of the drug were investigated on a variety of biological characteristics of angiogenesis, namely, endothelial cell migration, endothelial cell growth, apoptosis, as well as capillary-like tube formation.

To assess the antiangiogenic effects of drugs previously reported to have potential as antiangiogenic agents, effects of mitomycin C and cytoskeletal-disrupting agents (2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole and cytochalasin D) were investigated on the same biological characteristics as bleomycin. The effects of bleomycin, mitomycin C, colchicine, 2-methoxyestradiol, taxol and vinblastine on tumour development were subsequently studied in a mouse haemangioma model.

1. Systemic toxicity after intralesional bleomycin (IB) therapy has not been previously reported in haemangioma patients. Nevertheless, the potential for bleomycin-induced pulmonary toxicity when used in the treatment of non-malignant diseases remains a major concern. As an initial study into these potential side effects, the determination of circulatory spill-over after intralesional bleomycin therapy of haemangioma was considered imperative. In this study, a validated rapid and sensitive HPLC method for the detection of both major fractions of bleomycin, bleomycin A_2 and B_2 in human plasma was developed. The method's applicability in monitoring drug levels in patients receiving bleomycin was determined in samples of haemangioma patients treated with intralesional bleomycin, and in patients with various malignancies treated with intravenous bleomycin.

Bleomycin levels in plasma samples of haemangioma patients treated with IB were barely detectable over the course of 24 hours, with the highest plasma levels measured in any of the patients occurring one hour following IB treatment. In marked contrast, relatively high plasma bleomycin levels were detected in samples of cancer patients receiving intravenous bleomycin over the same time period. These findings suggest that the low levels of BLM detected in blood samples of IB treated haemangioma patients may translate to a reduced risk of developing pulmonary fibrosis.

2. Results from this study showed that bleomycin, 2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole and cytochalasin D inhibited of endothelial cell migration, although the anti-migratory effects of cytoskeletal-disrupting drugs were more pronounced. Mitomycin C on the other hand did not inhibit BME cell migration even at the highest dose. With respect to cell growth, results also showed that bFGF (10 ng/ml) had a stimulatory effect on BME cell growth. All test drugs, including mitomycin C inhibited BME cell growth in a dose-dependent manner, even in the presence of the angiogenic growth factor, bFGF. Of the DNA-disrupting drugs, mitomycin C inhibited BME cell growth with more potency than bleomycin. However, bleomycin inhibited endothelial cell migration.

It has been proven conclusively that endothelial cell migration and growth are central to the process of angiogenesis. Therefore, bleomycin, 2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole and cytochalasin D appear to have antiangiogenic activity at doses tested in this study. Although mitomycin C inhibited BME cell growth, the inhibition of cell growth is not a requisite of anti-angiogenic activity, since a number of compounds can exhibit antiangiogenic activity without exhibiting antiproliferative effects on endothelial cells. The effects of test drugs were then investigated using assays of apoptosis, since inhibition of this mode of cell death was associated with angiogenesis.

3. Inhibition of apoptosis (and the promotion of EC survival) has been cited as being important to the process of angiogenesis. Studies on BME cell morphology revealed that test agents induced apoptosis in these cells. These observations were confirmed using DNA fragmentation assay and acridine orange staining. Also, previous studies have shown that proangiogenic cytokines act by inhibiting apoptosis, and that deprivation of endogenous bFGF, which leads to the induction of endothelial cell apoptosis, resulting in inhibition of VEGF-induced *in vitro* angiogenesis. Results from this study therefore further indicate that these drugs may have antiangiogenic activity since they induced endothelial cell apoptosis. It was thus imperative that test drugs were evaluated for antiangiogenic activity in a 3-dimensional assay of *in vitro* angiogenesis.

4. In this study, bFGF induced BME cells grown on a 3-D matrix of collagen gel to form an extensive network of capillary-like tubes. BME cells to invade the underlying collagen gel matrix an extensive network of capillary-like tubular structures, a phenomenon that closely mimics the angiogenic process occurring *in vivo*.

Bleomycin, mitomycin C, 2-methoxyestradiol, taxol, vincristine, vinblastine, colchicine, nocodazole and cytochalasin D inhibited endothelial cell invasion of the underlying collagen gel, and formation of capillary-like structures. These findings therefore showed that test drugs inhibit angiogenesis *in vitro*.

5. Endothelioma (sEnd.3) cells injected into histocompatible mice rapidly organized to form vascular tumours at the site of injection with 100% frequency. Each of the blood parameters studied, namely, red cell count, haemoglobin concentration, hematocrit, and platelet counts were decreased. As the haemangioma increased in size in these sEnd.3 inoculated mice, the mice developed severe thrombocytopenia and anaemia.

Currently, there is no *in vivo* model that 'faithfully recapitulates the pathophysiology of infantile haemangiomas', and according to Pepper (1997), of the animal models which utilize transformed endothelial cells, the PymT model has been the most extensively characterized. Furthermore, the PymT model serves as a good functional model of haemangiomas for the following reasons: (i) the tumours are endothelial specific and organ non-specific; (ii) there is frequent involvement (including compression) of vital organs; and (iii) mice develop features of the Kasabach-Merritt syndrome, including sequestration of platelets. Therefore, further studies on the effects of various test drugs were undertaken using this model.

Bleomycin, mitomycin C, colchicine, 2-methoxyestradiol, taxol and vinblastine effectively inhibited tumour growth in the mice, and the reduction in tumour growth correlated with recovery from anaemia and thrombocytopenia.

Findings from the present study have indicated that DNA-damaging agents, bleomycin and mitomycin C, and the cytoskeletal disrupting agents tested, inhibit angiogenesis *in vitro* at different levels. However, cytoskeletal-disrupting agents were more potent in inhibiting the various aspects of angiogenesis than the DNA-damaging agents. The vinca alkaloids, vincristine and vinblastine, were the most potent drugs in inhibiting angiogenesis *in vitro*. However, vincristine was omitted from *in vivo* studies due to side effects observed at doses between 0.5 and 1 mg/kg in multiple preliminary studies.

Vinblastine, on the other hand, inhibited haemangioma development at 10x (10 mg/kg) the dose used with vincristine, and still did not induce any observable side effects. Although the effectiveness of vincristine in haemangioma treatment has been reported in a few cases, concerns over its debilitating side effects persist.

Vinblastine may thus offer an alternative treatment option for the tumours due to its similarity in structure to vincristine, it's more pronounced effectiveness in inhibiting angiogenesis *in vitro* and its apparent lack of side effects observed in *in vivo* studies employing a murine haemangioma model. Another drug, taxol, exhibited antiangiogenic activity (inhibition of capillary morphogenesis) at doses that were far lower that those inhibiting cell growth and inducing apoptosis. Again the implication is that this drug may be used clinically to treat haemangiomas at doses well below the tolerated dose. Indeed taxol has been shown to inhibit tumour angiogenesis *in vivo* at doses below the maximum-tolerated dose.

It was also shown in this study that bleomycin, mitomycin C and microtubule-disrupting drugs, colchicine, 2-ME, and taxol inhibited haemangioma development *in vivo*. This inhibition of tumour development was associated with an improvement in platelet numbers. According to the observations of Enjolras (1997) of patients with vascular tumours associated with KMS, when cessation of platelet consumption was achieved in such patients, the tumour mass resolved and patients entered a state of '*biologic and clinical remission*'. Indeed, in this study improved platelet numbers correlated with a reduction in tumour size.

The potential application of these angiogenesis inhibitors warrants their further study on this haemangioma model focussing on dose response and drug combinations to enhance their application singly (monotherapy) or to elaborate new therapeutic schedules and combination strategies for haemangioma treatment.

Future Research

The following areas have been identified for further studies:

- Analysis of bleomycin levels in patient samples needs to be undertaken using larger sample sizes (patient numbers), to confirm observations from the current study which showed negligible spill-over of the drug following intralesional injection. Since more than 70% bleomycin is excreted through the kidneys within 24 hours, these studies should be coupled with the determination of creatinine levels (which is a fairly reliable indicator of kidney function).
- Flagellate pigmentation is a side-effect associated with intralesional bleomycin treatment. Concerns have been raised about this side-effect being an early indicator of systemic toxicity, while other reports have attributed this side-effect to the induction of local inflammatory reaction by the drug. Further studies need to be undertaken to determine if there is a correlation between the dose of bleomycin injected, systemic bleomycin levels, and the occurrence of this side-effect.
- More recently, forms of programmed cell death, apart from apoptosis, have been implicated in the development of some tumours. The roles of these other forms of programmed cell death in angiogenesis and in haemangioma growth and involution were identified as another area which requires further investigation, as these other cell death pathways can serve as potential targets for antiangiogenic therapy.
- Based on reviewed literature, the effects of microtubule-disrupting drugs on circulating endothelial progenitor cells (CEPs) remain unclear. Circulating endothelial progenitor cells are thought to contribute to the development of haemangiomas, therefore, the effects of test drugs in inhibiting the mobilization or in reducing the viability of CEPs needs to be investigated.
- Various doses of the tested drugs should be investigated on the mouse haemangioma model, and their effects on tumour size, haematological parameters, in particular platelet counts should be correlated to determine the most effective drug dosages. Taking into consideration the fact that test drugs have different modes of biologic action, combinations of these drugs warrant investigation to determine the potential for better therapeutic outcome.

Appendix I. Factors that regulate normal angiogenesis

Factor	Biological Actions
VEGF	Increases endothelial cell permeability
	Stimulates endothelial cell uPA/PAI-1 production
	Stimulates endothelial cell proliferation
	Inhibits endothelial cell apoptosis
	Enhances endothelial cell migration
	Stimulates in vivo angiogenesis
Ang1	Stimulates in vitro endothelial cell sprout formation
	Increases girth and stability of endothelium
Ang2	Antagonizes Ang1 signalling/destabilizes endothelium
aFGF bFGF	Stimulates endothelial cell proliferation
	Enhances endothelial cell migration
	Stimulates endothelial cell PA/collagenase production
	Stimulates endothelial cell tube formation
	Stimulates in vivo angiogenesis
PDGF	Stimulates DNA synthesis in endothelial cells
	Stimulates endothelial cells to form chords in vitro
	Stimulates proliferation of smooth muscle cells and pericytes
	Induces vWF, VEGF, and VEGFR-2 expression in cardiac endothelial cells
	Increases capillary wall stability
TGF-β	Supports anchorage-independent growth of fibroblasts
	Inhibits proliferation and migration of endothelial cells
	Stimulates/inhibits formation of endothelial cell tubes in vitro
	Produces net antiproteolytic activity via modulation of uPA/PAI-1 expression levels
	Inhibits production of other proteases/stimulates production of protease inhibitors
	Stimulates VSMA production by pericytes
	Chemotactic for monocytes and fibroblasts
	Stimulates in vivo angiogenesis in presence of inflammatory response
	Increases vessel wall stability
TNF-α	Stimulates angiogenesis in vivo
	Stimulates formation of endothelial cell tubes in vitro
	Inhibits endothelial cell proliferation
EGF, TGF-α	Stimulate endothelial cell proliferation
	Stimulate angiogenesis in vivo
G-CSF, GM-CSF	Stimulate endothelial cell proliferation and migration

Angiogenin	Stimulates angiogenesis in vivo
	Supports endothelial cell binding and spreading
Angiotropin	Stimulates random capillary endothelial cell migration
	Stimulate endothelial cell tube formation
	Stimulates in vivo angiogenesis
Tissue factor	Contributes to development of yolk sac vasculature
Factor V	Contributes to development of yolk sac vasculature
Prostaglandin	Stimulates in vivo angiogenesis
Nicotinamide	Stimulates in vivo angiogenesis
Monobutyrin	Stimulates in vivo angiogenesis
	Stimulates endothelial cell migration in vitro
Membrane-bound proteins	
$\alpha_v\beta_3$-Integrin	Highly expressed on activated endothelial cells
	Mediates endothelial cell attachment, spreading, and migration
	Present on angiogenic capillary sprouts
	Required for bFGF-stimulated angiogenesis in vivo
	Localizes MMP-2 to capillary sprouts
	Suppresses endothelial cell apoptosis
$\alpha_v\beta_5$-Integrin	Required for VEGF-stimulated angiogenesis in vivo
α_5 $_1$-Integrin	Required for non-VEGF growth factor-stimulated angiogenesis in vivo
VE-cadherin	May mediate permeability of endothelium
	Required for in vivo angiogenesis
	Prevents endothelial cell apoptosis
Eph-4B/Ephrin-B2	Colocalize at venous/arterial interfaces of developing embryo
	Required for angiogenesis of head and yolk sac and for myocardial trabeculation.
Ephrin-A1	Required for in vivo angiogenesis induced by TNF-
	Chemotactic for endothelial cells in vitro
Eph-2A	Required for endothelial cell tube formation in vitro
Biomechanical forces	
Blood flow/shear stress	Increases endothelial stress fiber formation (if laminar)
	Promotes endothelial cell division (if turbulent)
	Stimulates transcription of bFGF and TGF- genes

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Appendix II. Factors that regulate tumour angiogenesis

Factor	Role in Tumor Neovascularization
VEGF	Secreted by many tumor cells in vitro
	Highly upregulated in most human cancers
	Expression correlates with intratumoural microvessel density and poor prognosis in cancer patients
	Inhibition decreases tumor vessel density and tumor growth
FGF	Inhibition suppresses generation of tumor vessels in vitro and in vivo and tumor growth in vivo
	Important for maintenance, vs. induction, of tumor angiogenesis
	Synergizes with VEGF to promote angiogenesis in vitro and in vivo
	Induces VEGF expression in tumor cells and VEGF receptor expression in endothelial cells
Heparanase	Stimulates invasion and vascular sprouting of endothelial cells
	Releases bFGF from extracellular matrix
	mRNA and protein are enriched in metastatic tumor cell lines and human tumors vs. normal tissues
	Overexpression renders nonmetastatic cell lines metastatic in vivo and increases tumor neovascularization
Ang 2	Induced in endothelial cells of preexisting vessels co-opted by a tumor, leading to vessel regression
	Induced in endothelial cells of newly formed vessels of tumor, leading to vessel plasticity and VEGF-mediated growth
IL-8	Mitogenic and chemotactic for HUVECs in vitro
	Stimulates angiogenesis in vivo
	mRNA is upregulated in neoplastic tissues vs. normal ones in vivo; expression correlates with extent of neovascularization
	Overexpression increases invasiveness, tumourigenicity, neovascularization, and metastatic potential of tumor cells
	Mediates stimulation of MMP-2 gene transcription
MMP-2	Directly modulates melanoma cell adhesion and spreading on extracellular matrix
	Mediates tumor growth and neovascularization in CAM

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ADDENDUM I. List of congresses where parts of the work were presented.

1. **Mabeta P**. Shelver G and Dippenaar N. Development of an HPLC method for the determination of bleomycin profiles in urine. Paper at the International Immunopharmacology congress, Pilanesburg, South Africa. **2001**.

2. **Mabeta P,** Shelver G and Dippenaar N. An assay method for the determination of bleomycin profiles in hemangioma patients. Paper at the congress of the Physiology Society of Southern Africa. Stellenbosch, South Africa. **2002**.

3. **Mabeta P,** Shelver G, Dippenaar N, Davis PF. and Tan ST. Levels of bleomycin in haemangioma patients undergoing intralesional bleomycin injection (IBI). Poster at the congress of the International Society for the study of vascular anomalies. Wellington, New Zealand. **2004**.

4. **Mabeta P,** Soley JT and Davis PF. Assessment of the effects of bleomycin and interferon α-2a on angiogenesis in a human vascular tumour model. Paper presented at the congress of the Microscopy Society of Southern Africa. Port Elizabeth. **2006**.

5. **Mabeta P,** Davis PF. and Pepper MS. Interferon alpha and bleomycin exert antiangiogenic activity through different mechanisms. Poster at the Nature Biotechnology Winter Symposium. Miami, USA. **2006**.

6. **Mabeta P**, Pepper MS. The effect of Paclitaxel on neovessel formation *in vitro.* Poster at the congress of the Physiology Society of Southern Africa. Muldersdrift, South Africa. **2007**.

7. **Mabeta P,** Davis PF. and Pepper MS. Assessment of the mechanisms of antiangiogenic action of bleomycin and interferon alpha. Poster at the congress of the Physiology Society of Southern Africa. Muldersdrift, South Africa. **2007**.

ADDENDUM II. List of abstracts and articles published from this work.

1. **Mabeta P,** Soley JT and Davis PF. Assessment of the effects of bleomycin and interferon α-2a on angiogenesis in a human vascular tumour model. Abstract. Microscopy Society of Southern Africa – Proceedings 2006; 36:6.

2. **P. Mabeta**, P.F. Davis. The mechanism of bleomycin in inducing haemangioma regression. SAMJ 2008; 98:5389-539**.**

3**.** Ionescu G, **Mabeta P**, Dippenaar N, Muir T, Fourie P, Shelver G. Bleomycin plasma spill-over levels in paediatric patients undergoing intralesional injection for the treatment of haemangiomas. SAMJ 2008; 98:539-540.

ADDENDUM III. List of awards.

1. South African Women in Science Award, 2005 – Gender responsive research – Finalist (second place).

2. Microscopy Society of Southern Africa, 2006 – Innovative Research – 1st position.

3. Junior researcher of the year, 2007, Faculty of Health Sciences, University of Pretoria – 3rd Place.