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.

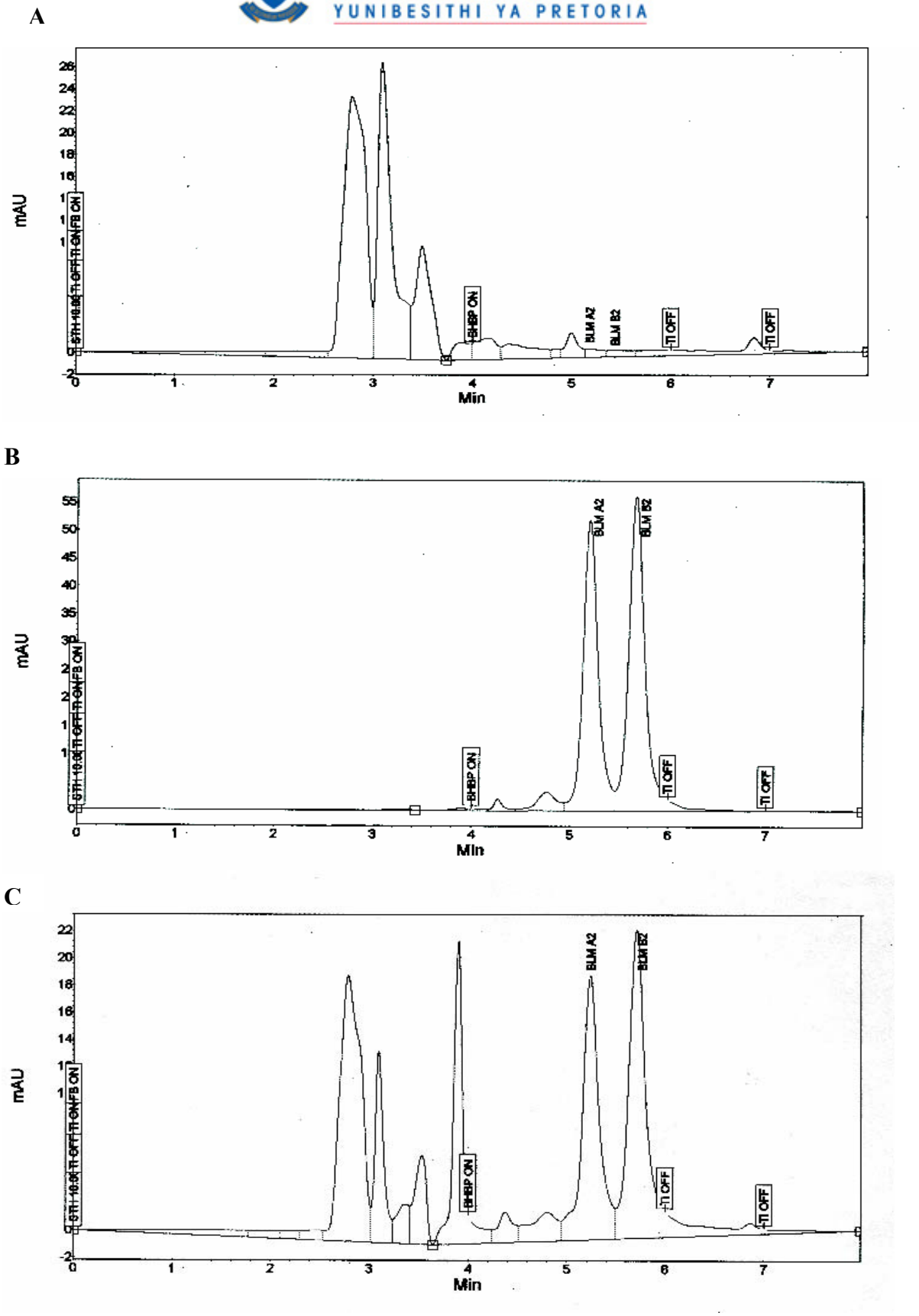
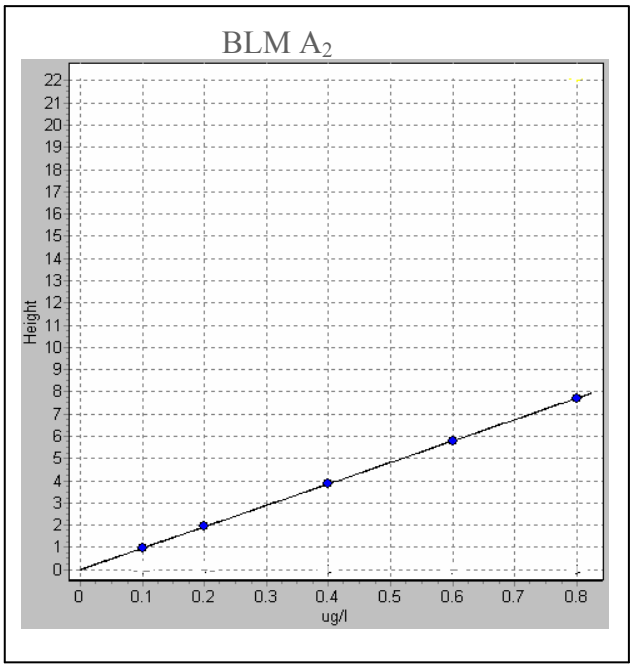
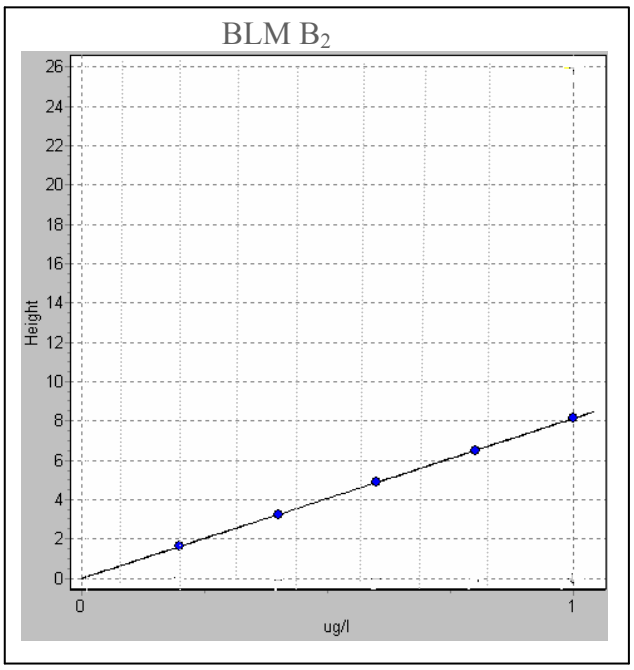


Figure 3.1. Chromatograms (absorbance in mAU versus time in minutes) obtained from the analysis of (A) Control human plasma, (B) Bleomycin stock in aqueous solution, and (C) Human plasma spiked with bleomycin A_2 (20 µg/ml) and bleomycin B_2 (30 µg/ml).



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

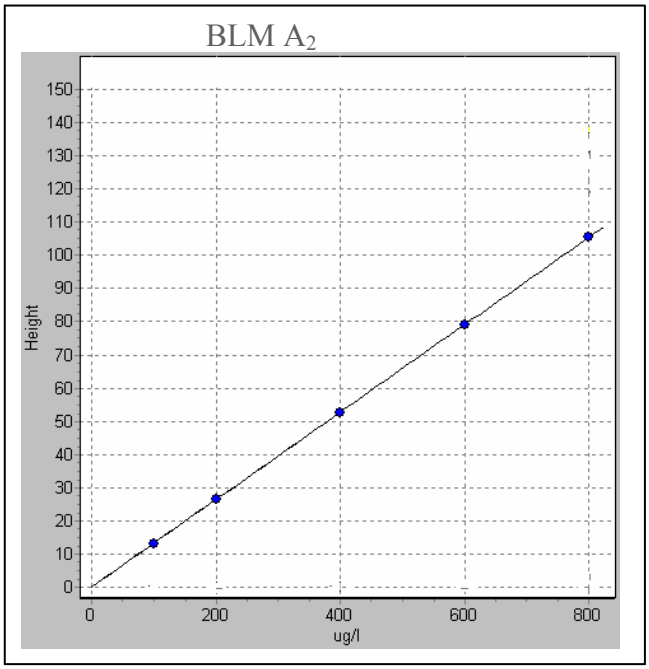
Y : Peak height (response)
X : BLM concentration



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

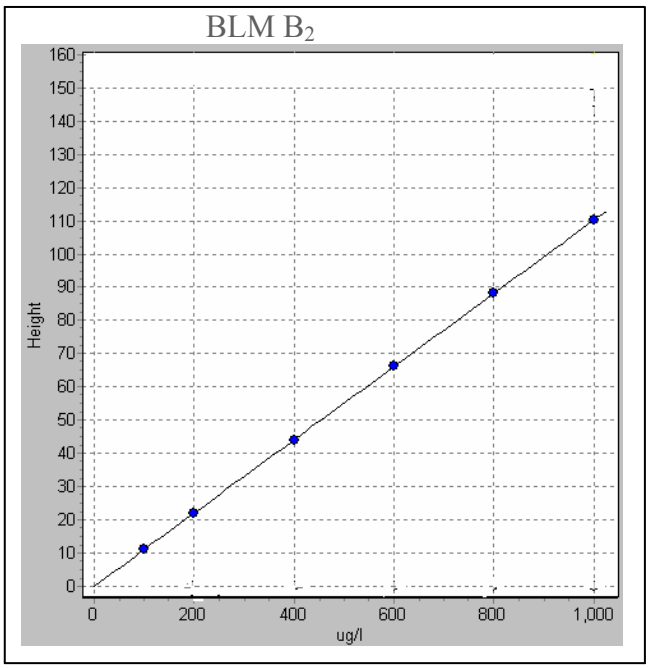
Y : Peak height (response)
X : BLM concentration (µg/ml)

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.

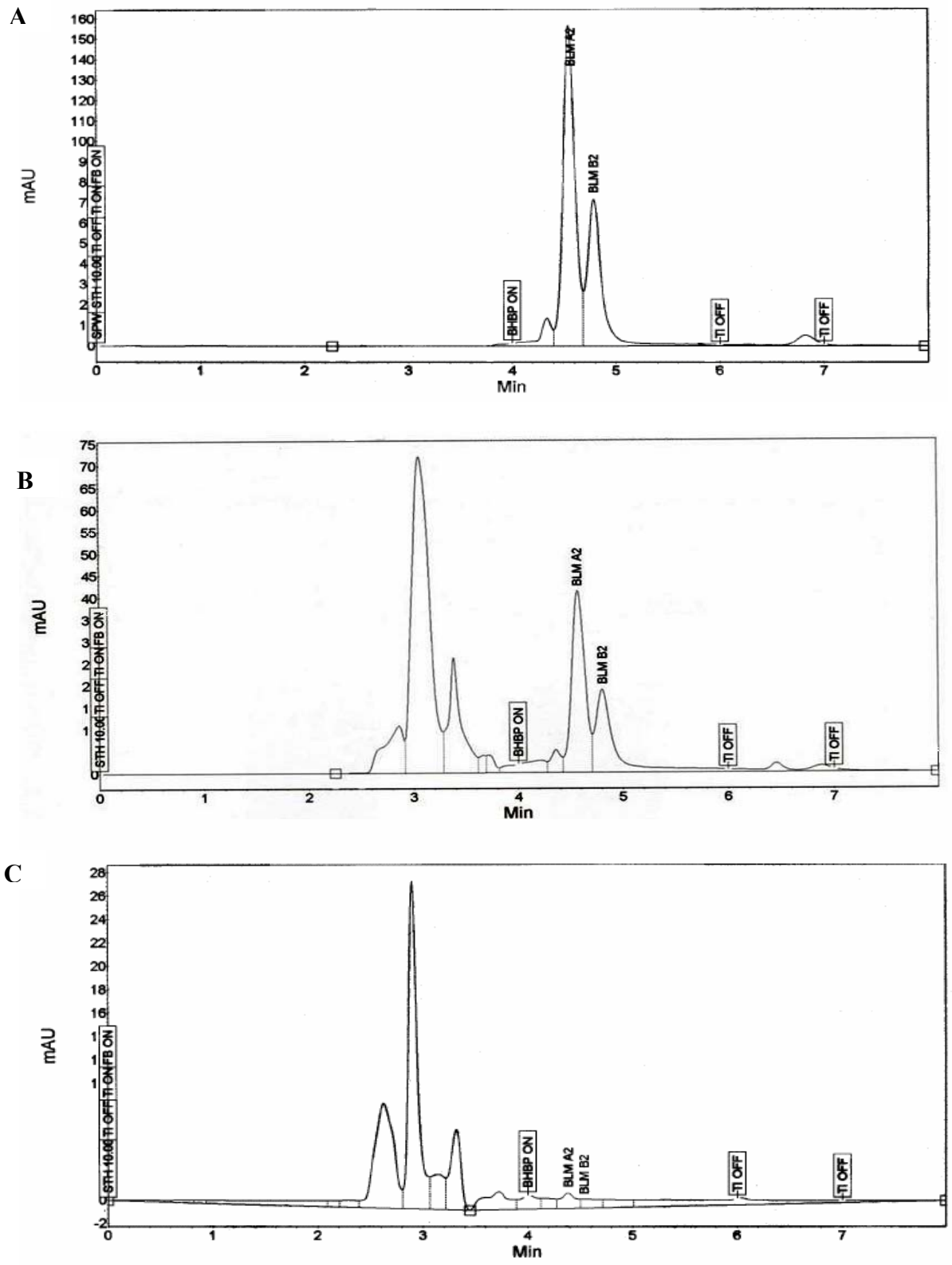


Figure 3.3. Chromatograms (absorbance in mAU versus time in minutes) obtained from the analysis of: (A) Blenoxane (clinically formulated bleomycin) reconstituted at 1mg/ml in sterile 0.9% saline, (B) a blood sample obtained from a lymphoma patient 1 hour after IV bleomycin treatment; (C) a blood sample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

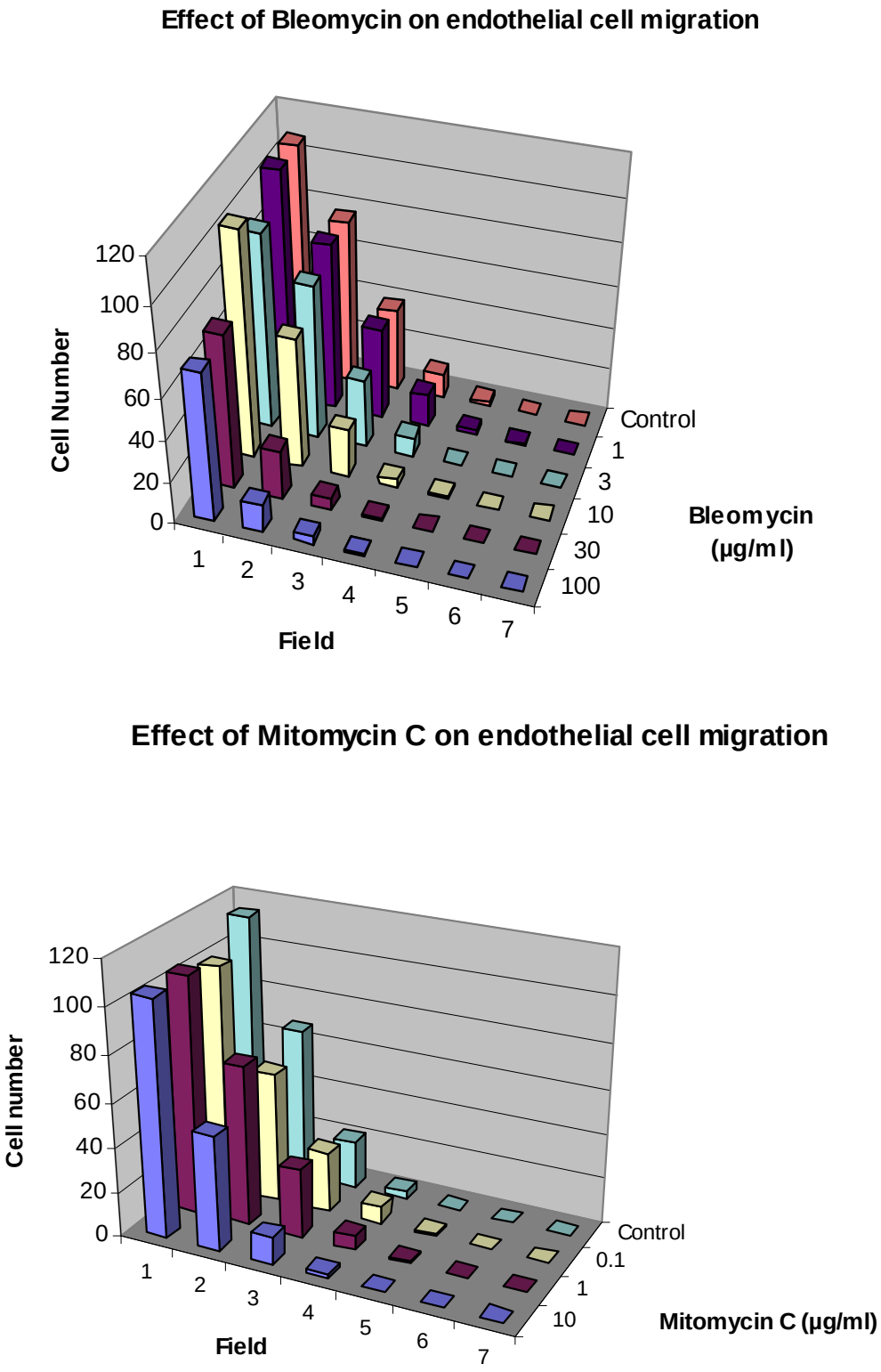


Figure 4.1. Effects of bleomycin and mitomycin C on endothelial cell migration.
Confluent BME cell monolayers were wounded with a blade so as to mark an initial wound edge and washed. Cells were then treated with saline (control), bleomycin or mitomycin C. After 15 hours, cultures were fixed and stained, 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.

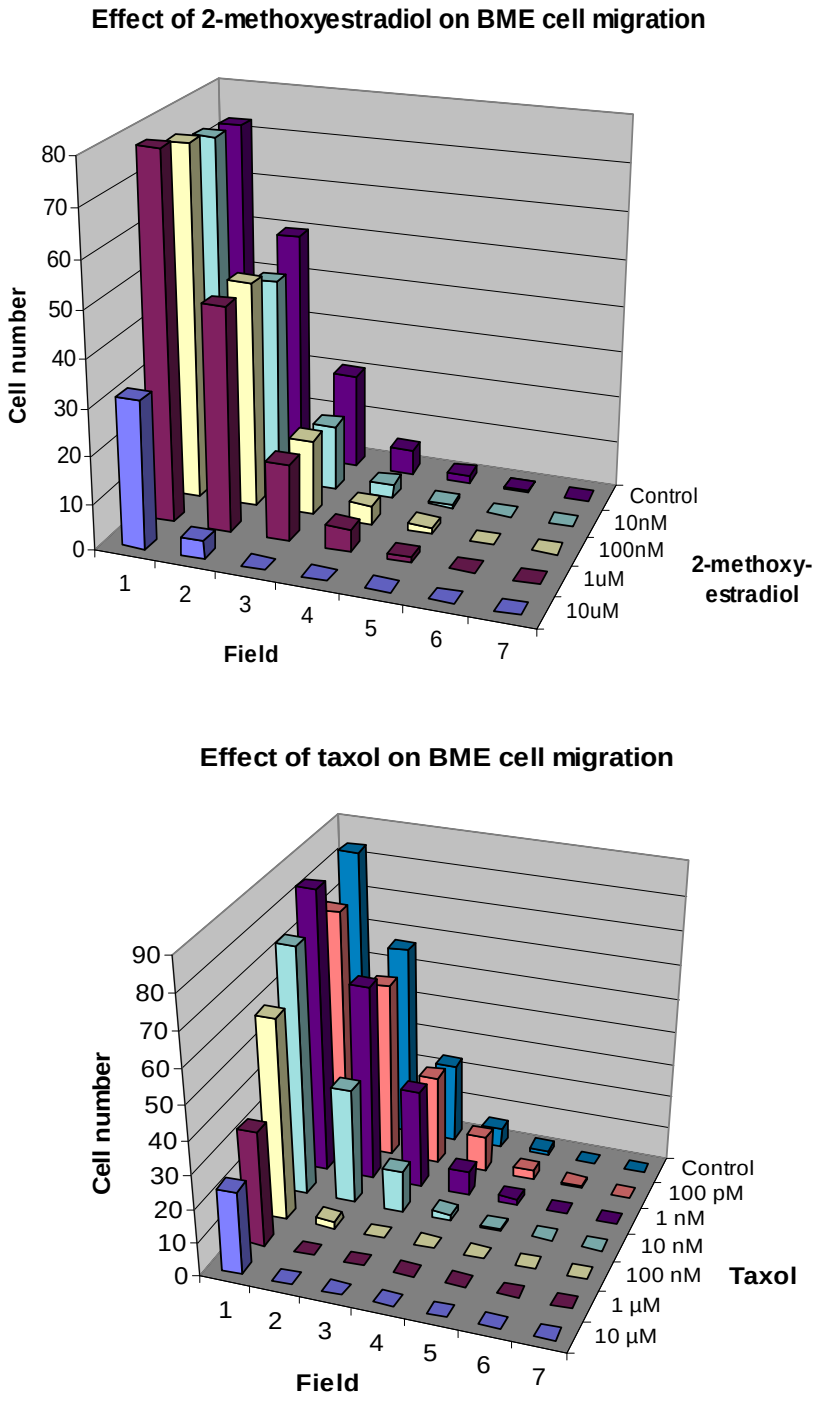


Figure 4.2. Effects of 2-methoxyestradiol and taxol on BME cell migration.
Confluent BME cell monolayers were wounded with a blade so as to mark an initial wound edge and washed. Cells were then treated with DMSO (control), 2-ME or taxol. After 15 hours, cultures were fixed and stained, and subsequently 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.

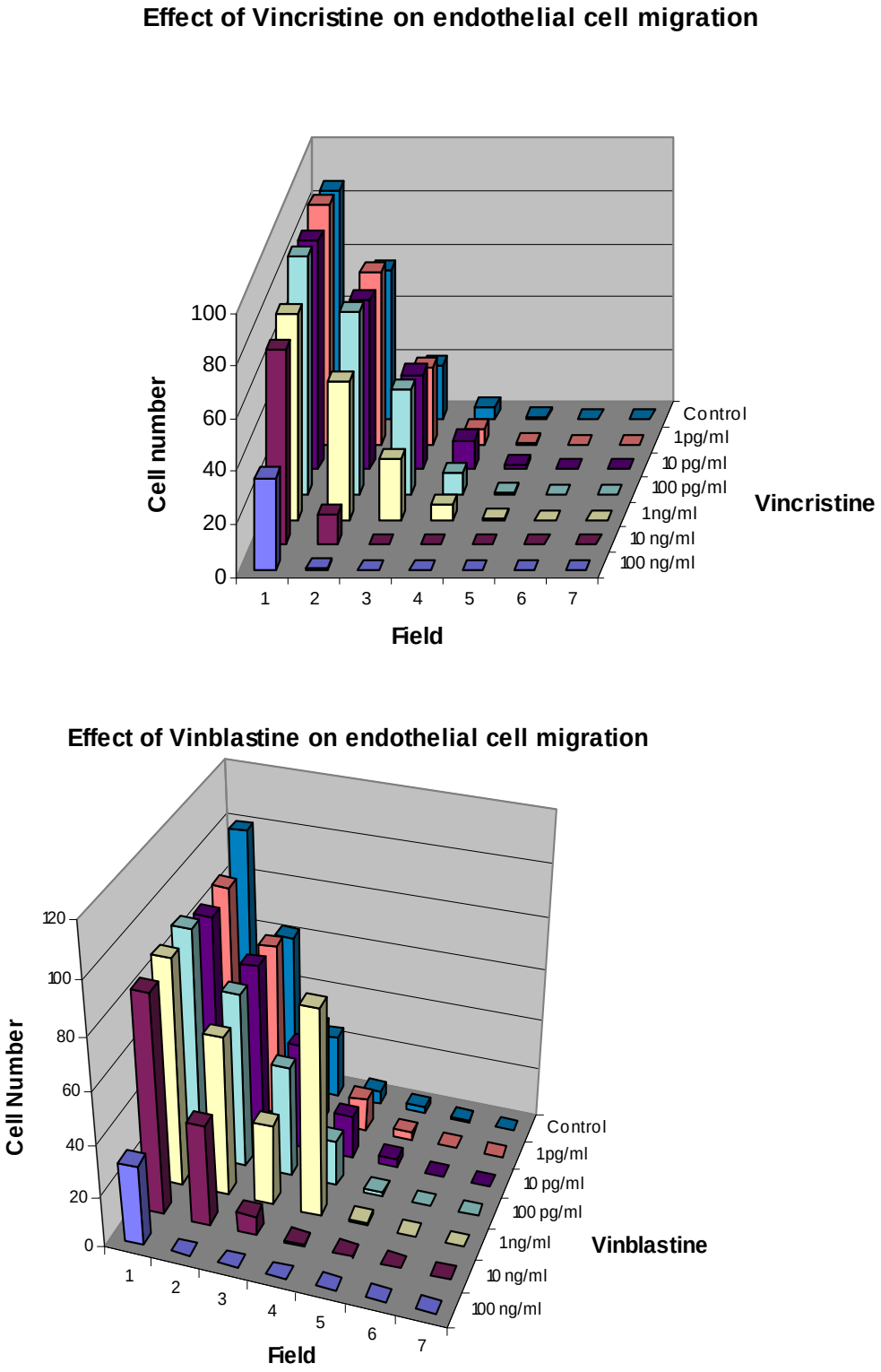


Figure 4.3. Effects of vincristine and vinblastine on endothelial cell migration.
Confluent BME cell monolayers were wounded with a blade so as to mark an initial wound edge and washed. Cells were then treated with saline (control), vincristine or vinblastine. After 15 hours, cultures were fixed and 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.

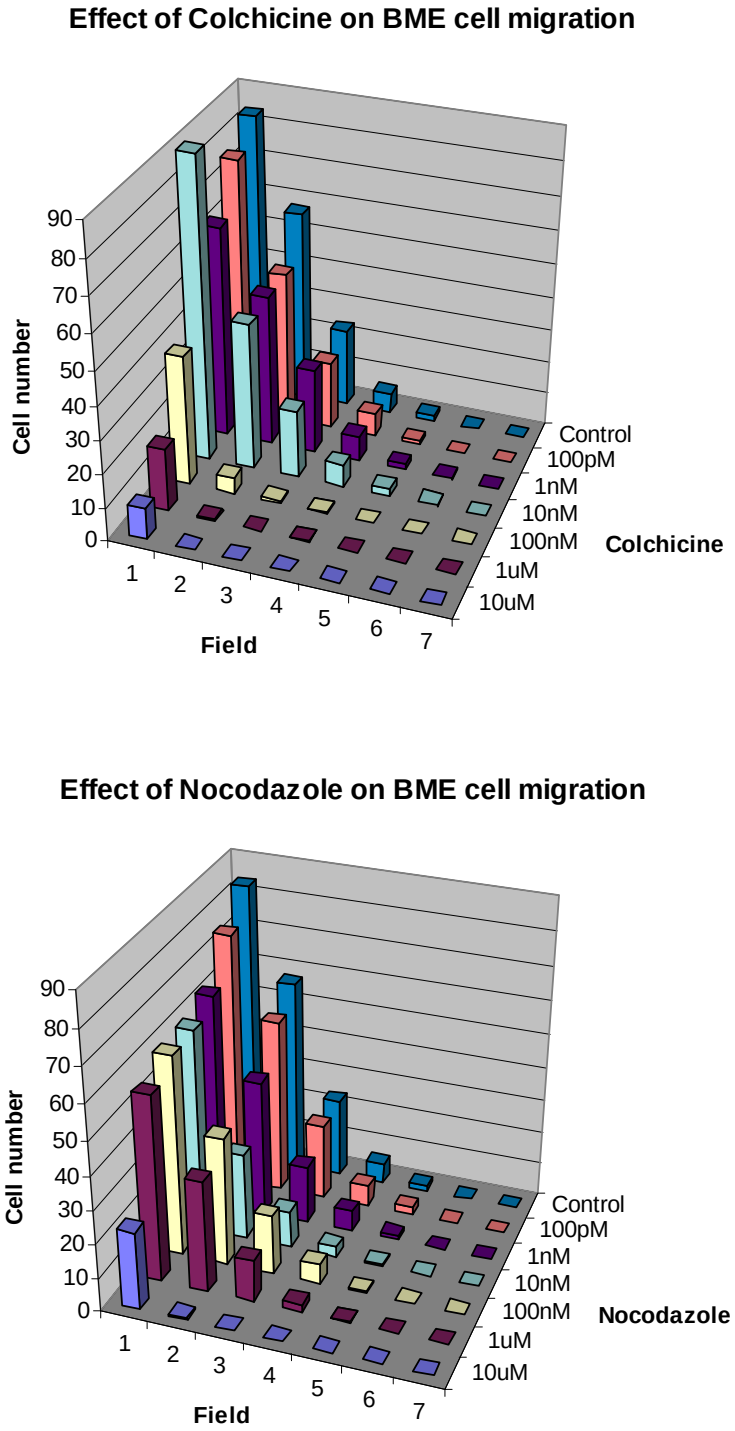


Figure 4.4. Effects of colchicine and nocodazole on BME cell migration.
Confluent BME cell monolayers were wounded with a blade so as to mark an initial wound edge and washed. Cells were then treated with DMSO (control), colchicine or nocodazole. After 15 hours, cultures were fixed and stained, and subsequently 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.

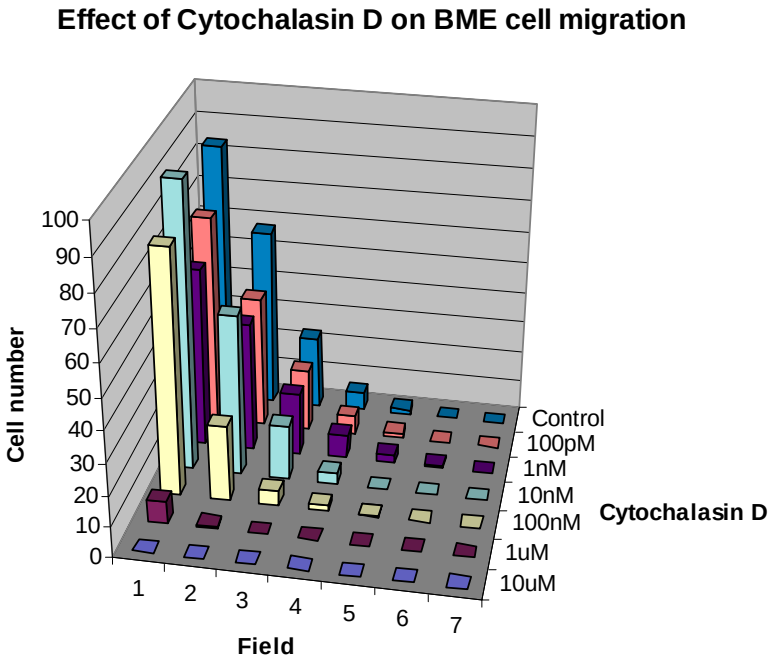
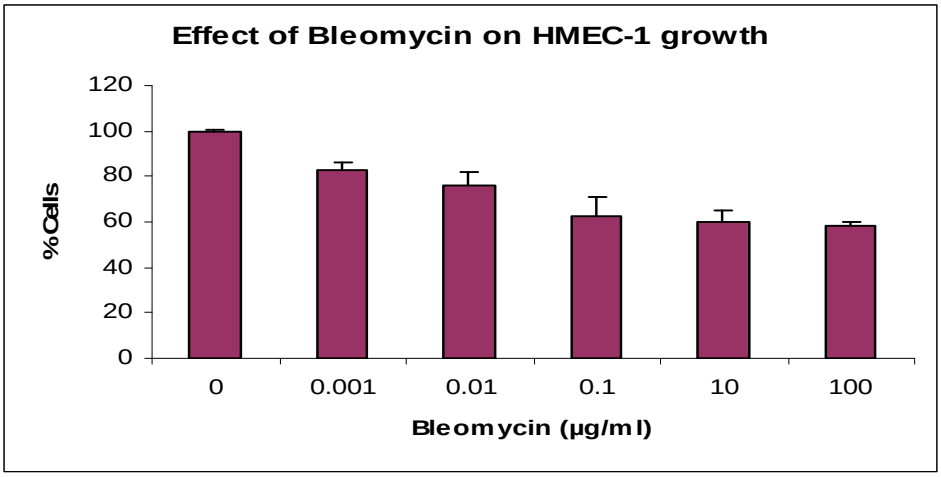
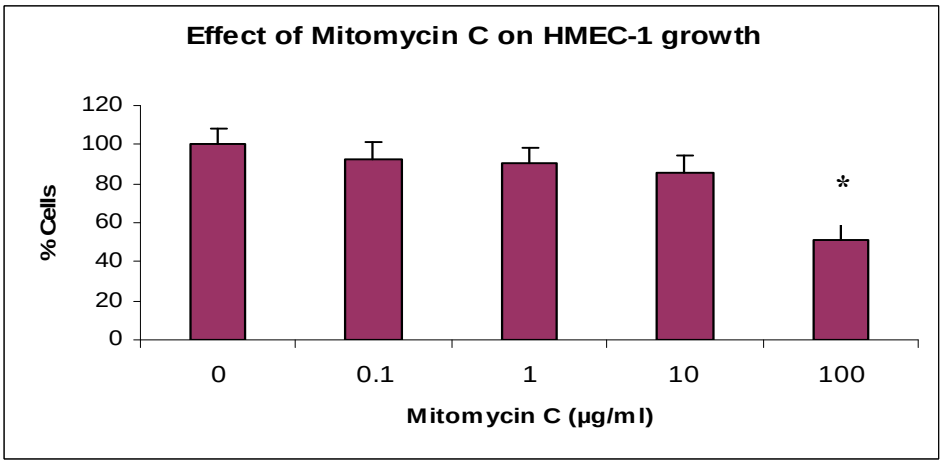


Figure 4.5. Effect of cytochalasin D on BME cell migration.
Confluent BME cell monolayers were wounded with a blade so as to mark an initial wound edge and washed. Cells were then treated with DMSO (control), cytochalasin D. After 15 hours, cultures were fixed and stained, and subsequently viewed with 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.

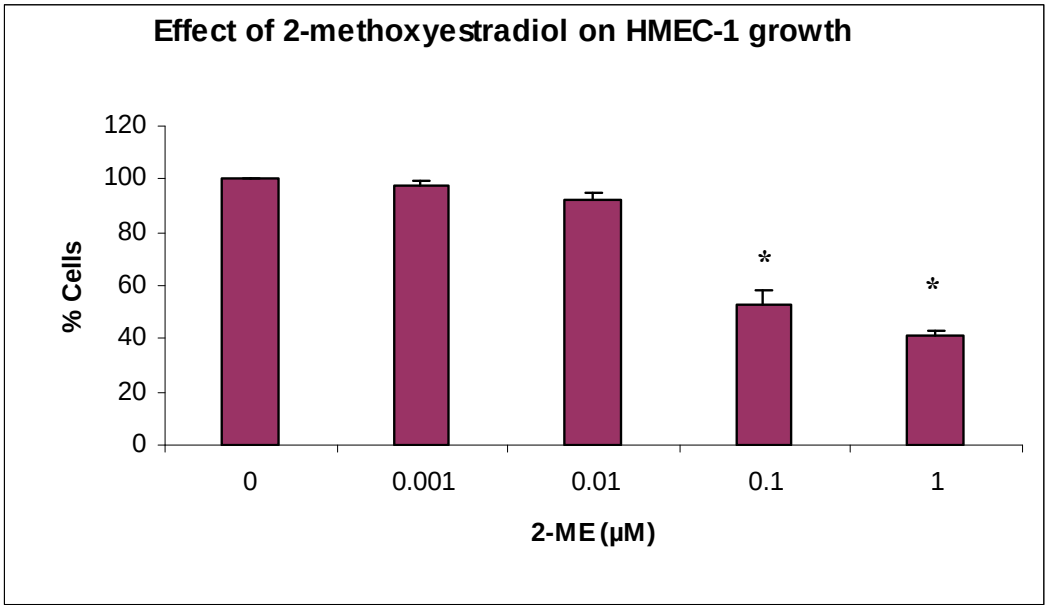


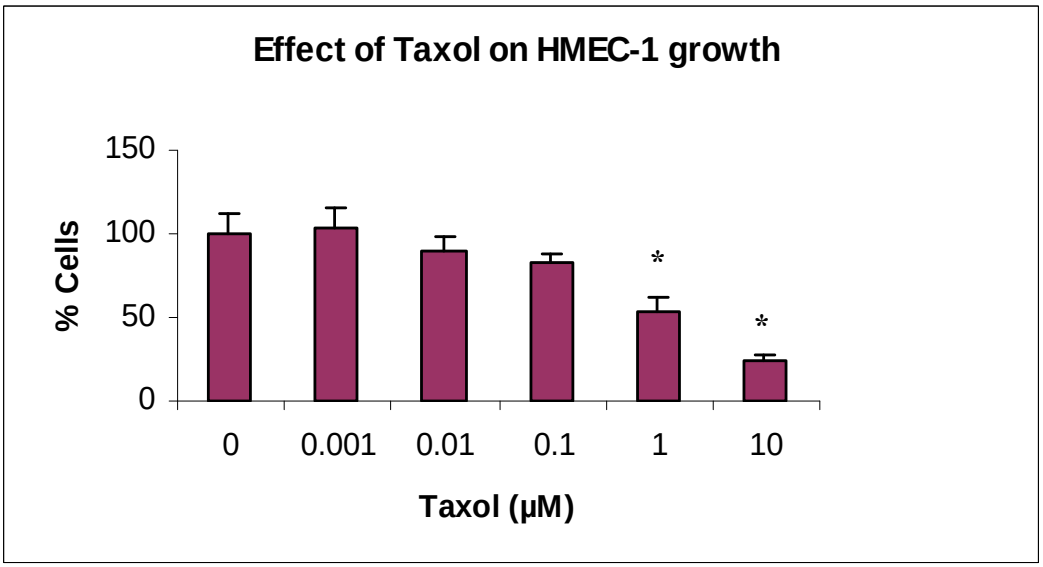


* Significant difference between the number of control and treated cells: $P < 0.05$

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.

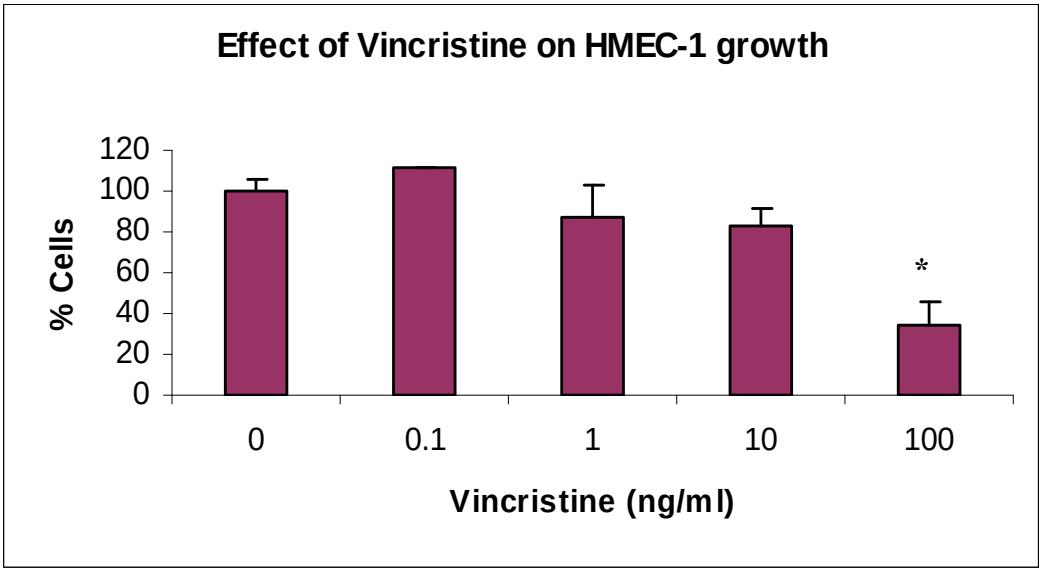


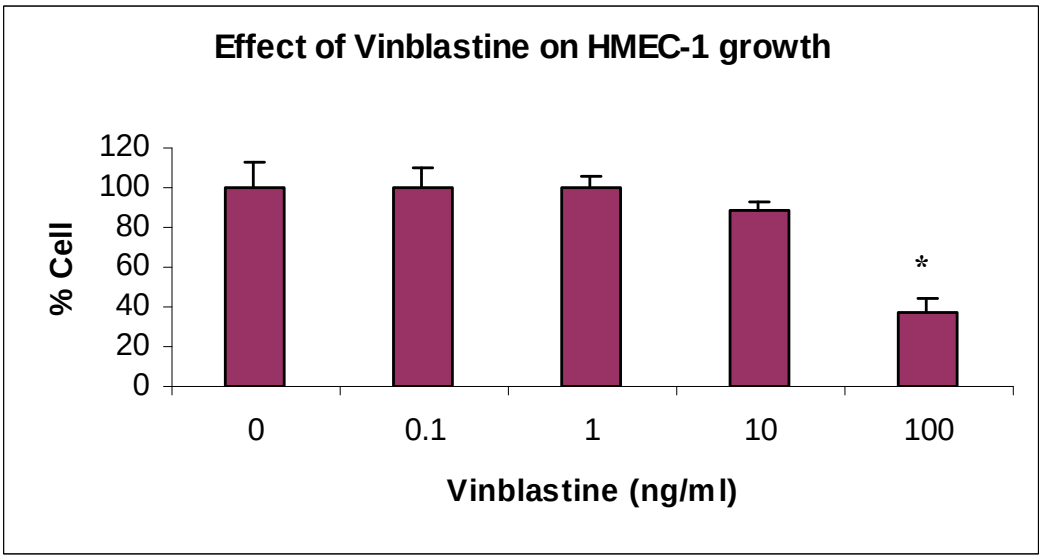


* Significant difference between the number of control and treated cells: $P < 0.05$

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.

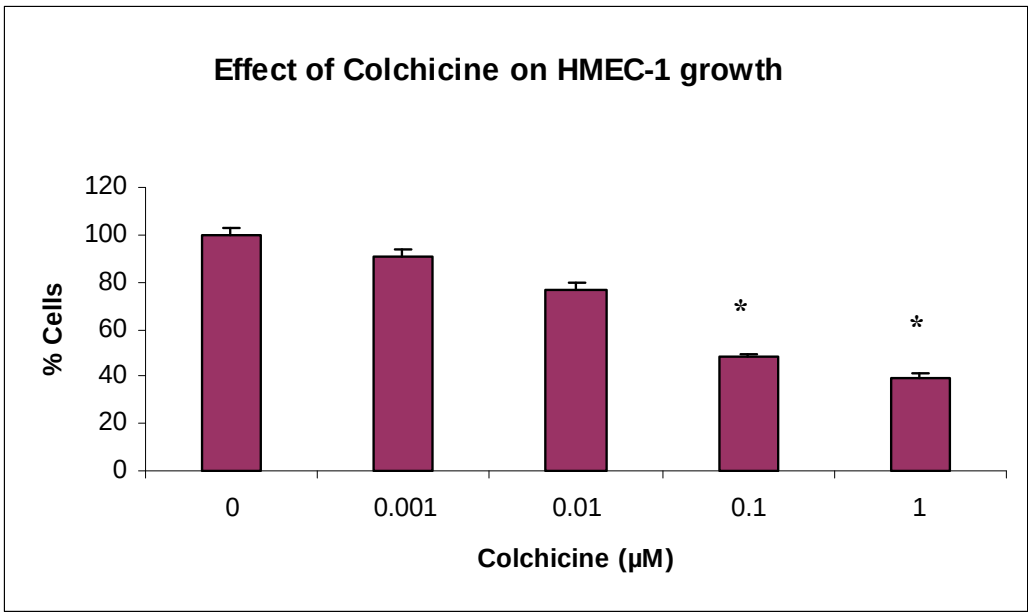


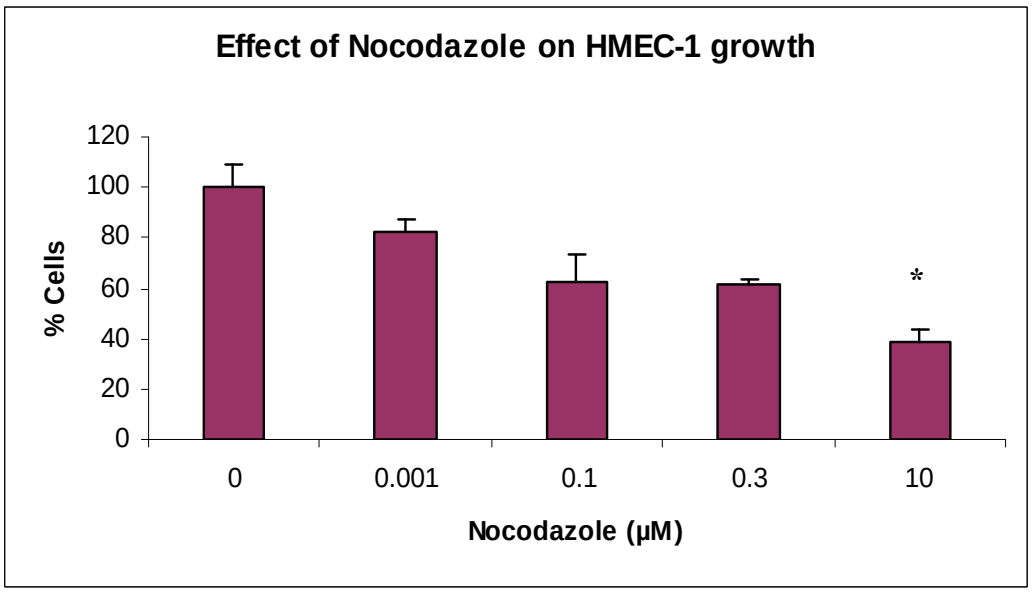


* Significant difference between the number of control and treated cells: $P < 0.05$

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.

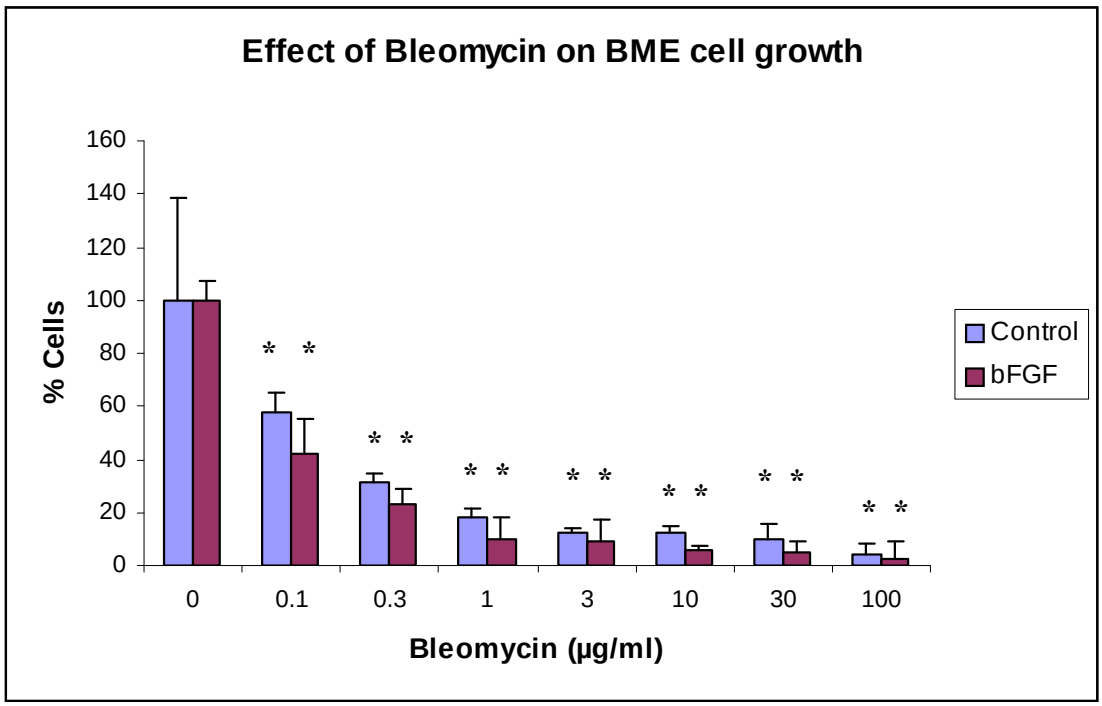


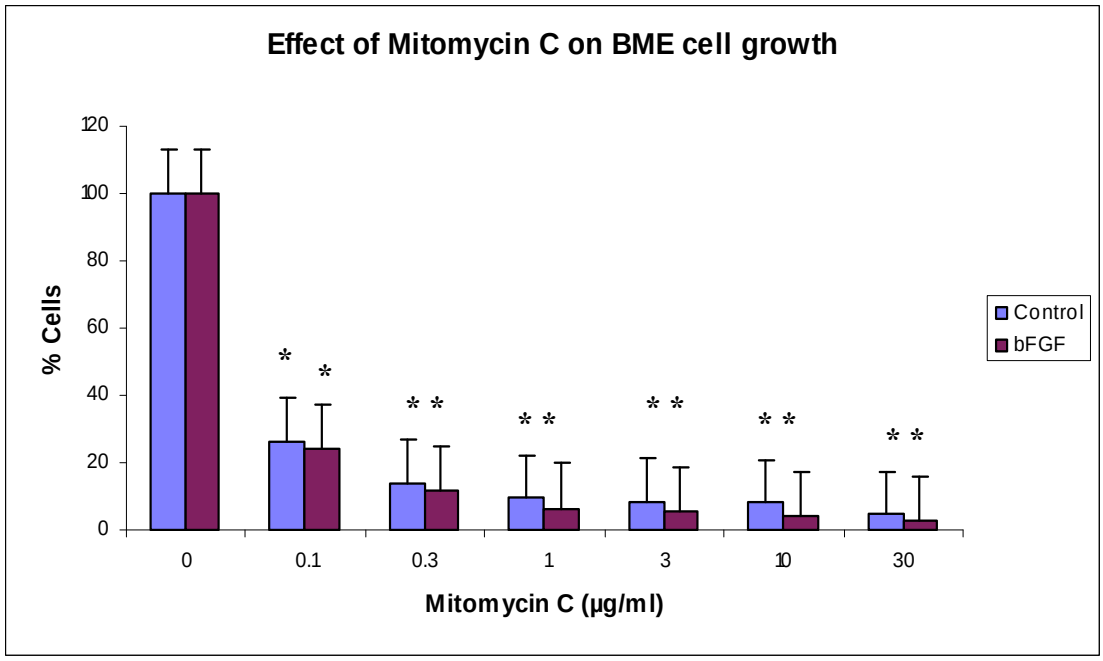


* Significant difference between the number of control and treated cells: $P < 0.05$

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.

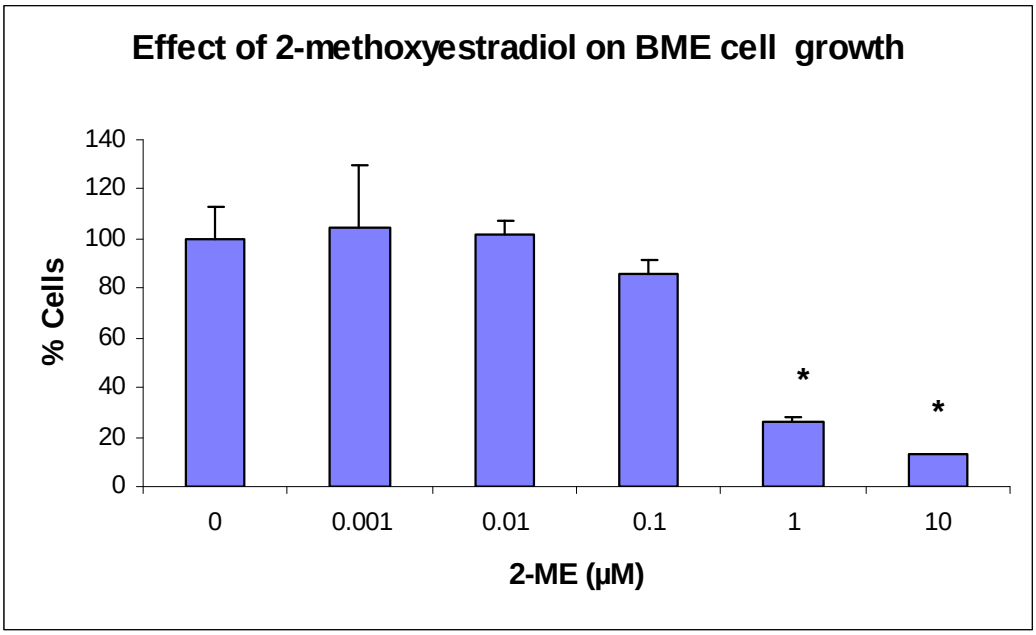


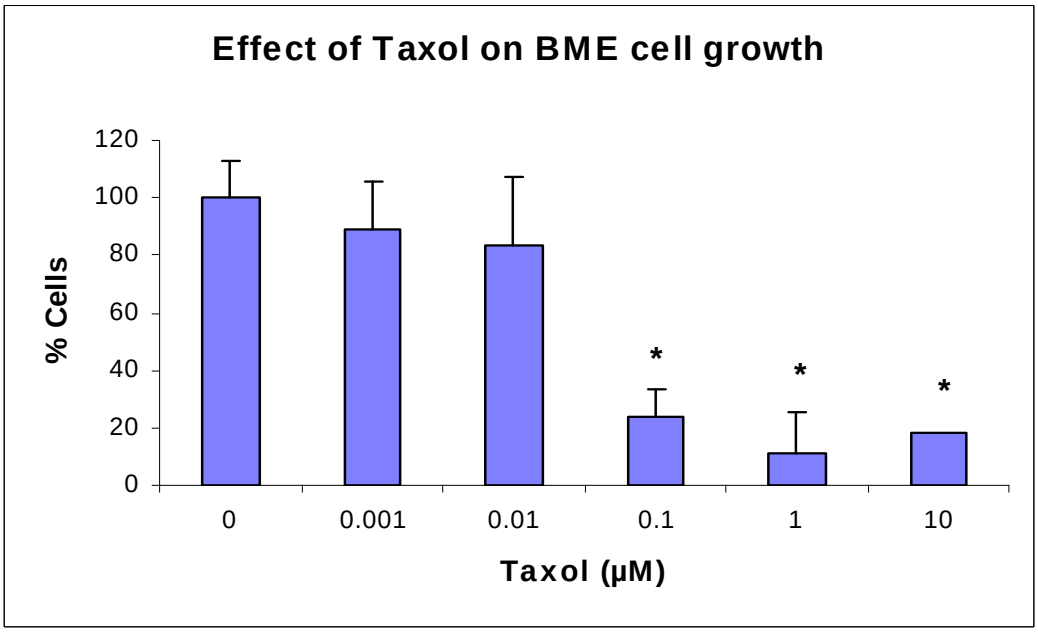


* Significant difference between the number of control and treated cells: $P < 0.05$

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.

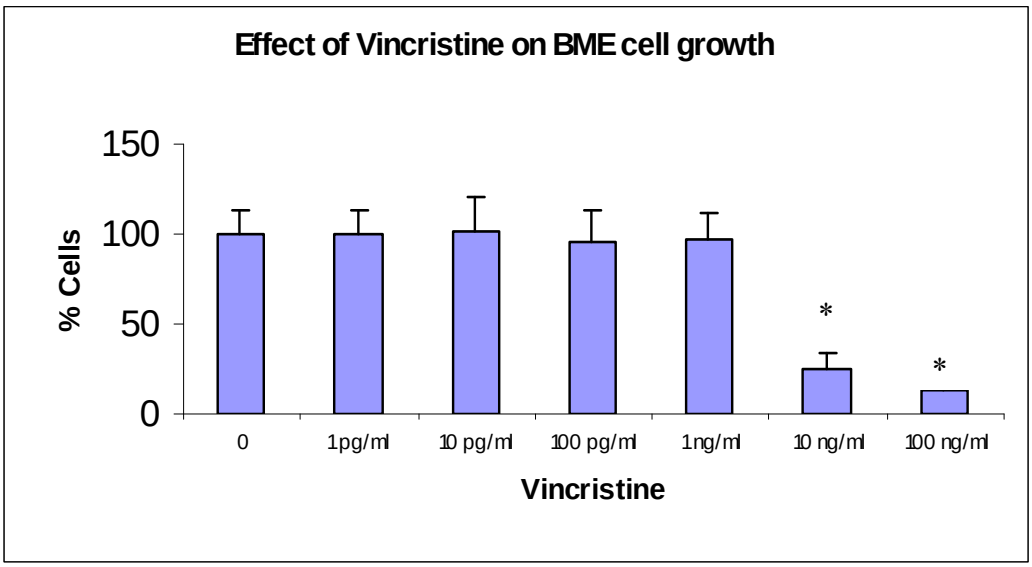


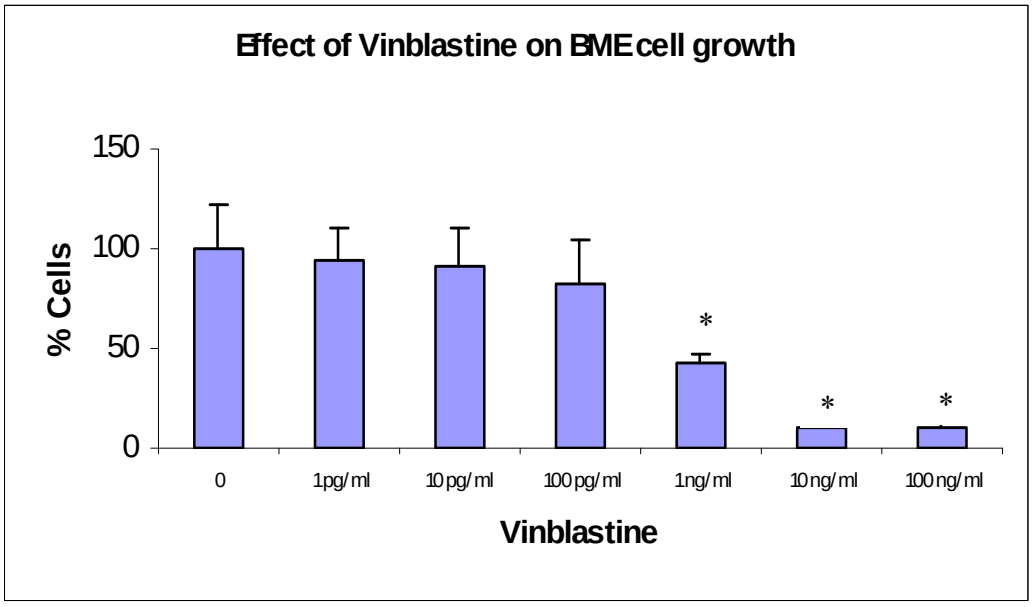


* Significant difference between the number of control and treated cells: $P < 0.05$

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.

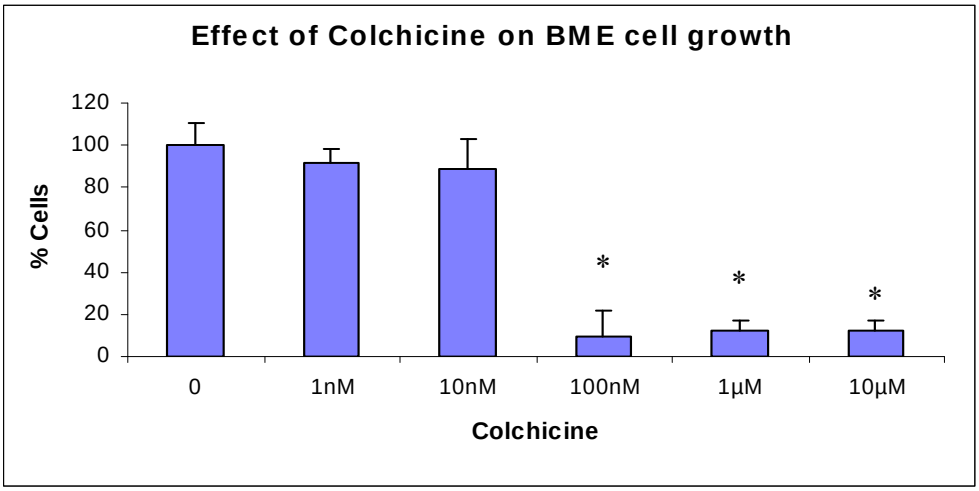


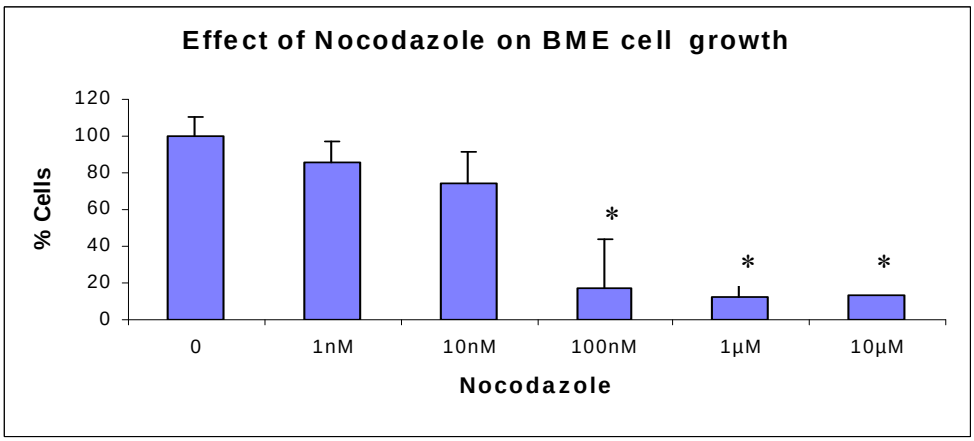


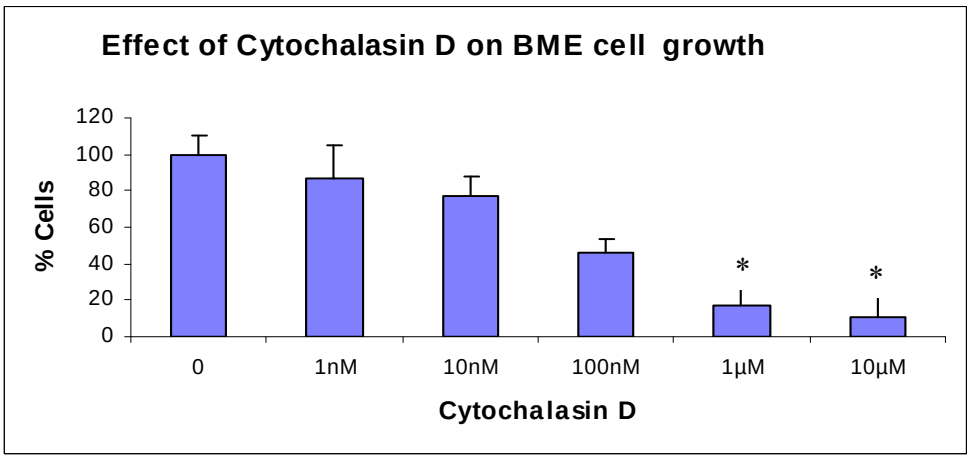
* Significant difference between the number of control and treated cells: $P < 0.05$

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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