

## 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.<sup>1</sup> 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.<sup>2,3</sup>

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.<sup>4</sup> The middle T antigen of murine polyoma virus rapidly transforms endothelial cells, leading to the formation of vascular tumours in newborn mice.<sup>4,5</sup>

*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.<sup>6</sup> On the other hand, normal endothelial cells grown on a physiologically relevant 3-D substratum form capillary-like tube structures.<sup>7,8</sup> *In vivo*, in several different species, End. cells rapidly induce haemangiomas.<sup>4</sup> 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 Dulbecco'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 °C incubator in a humidified atmosphere containing 5% CO<sub>2</sub>.

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.2 cell growth using crystal violet nuclear staining method as described by Gillies *et al.* (1983).<sup>9</sup>

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<sub>2</sub>O) 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).<sup>10</sup> 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  $2 \times 10^6$ . 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.

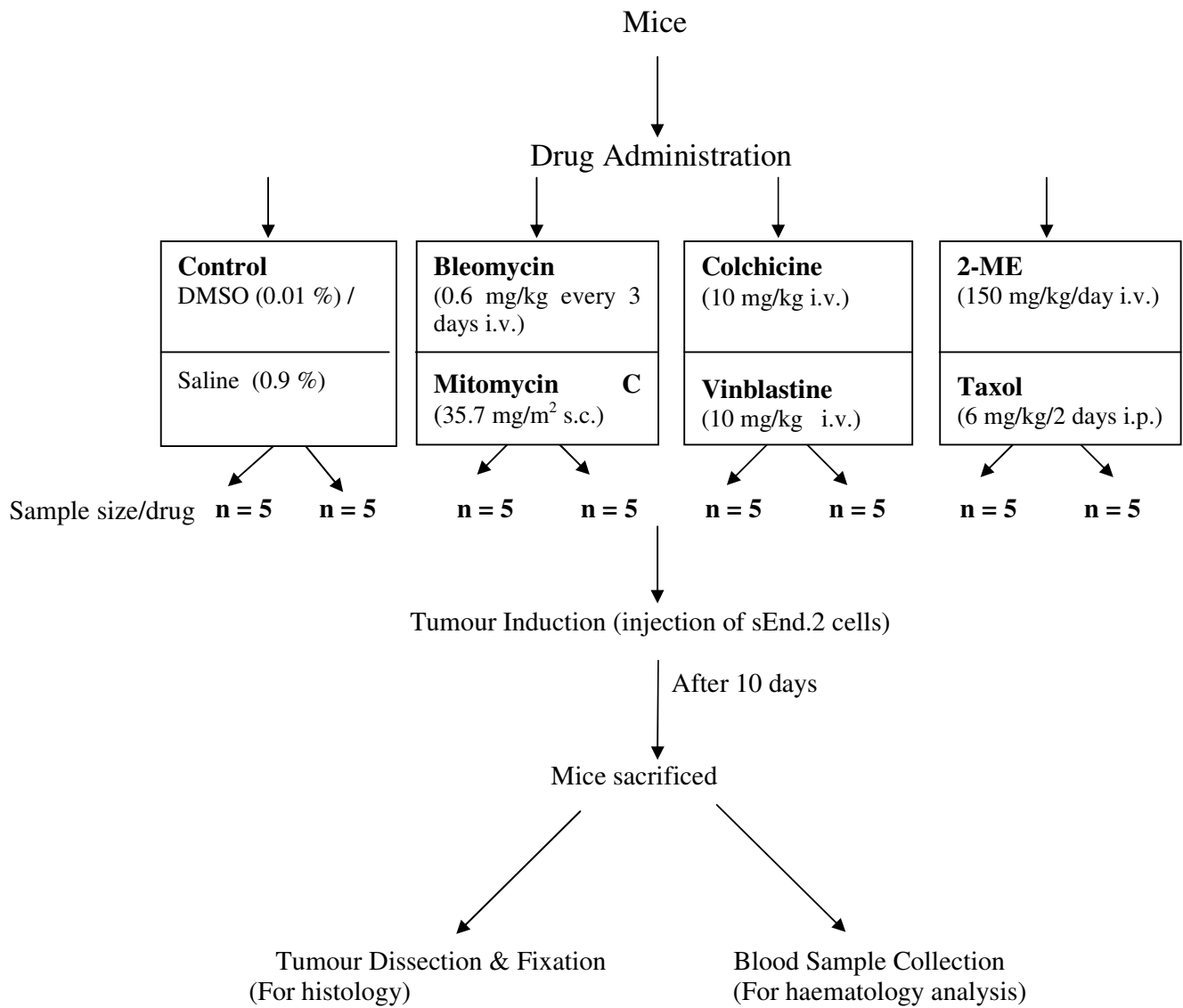


Figure 7.1. Schematic diagram of the experimental design.

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).

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

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  $\mu\text{g/ml}$  respectively. The  $\text{IC}_{50}$  values for the drugs are listed in table 7.2.

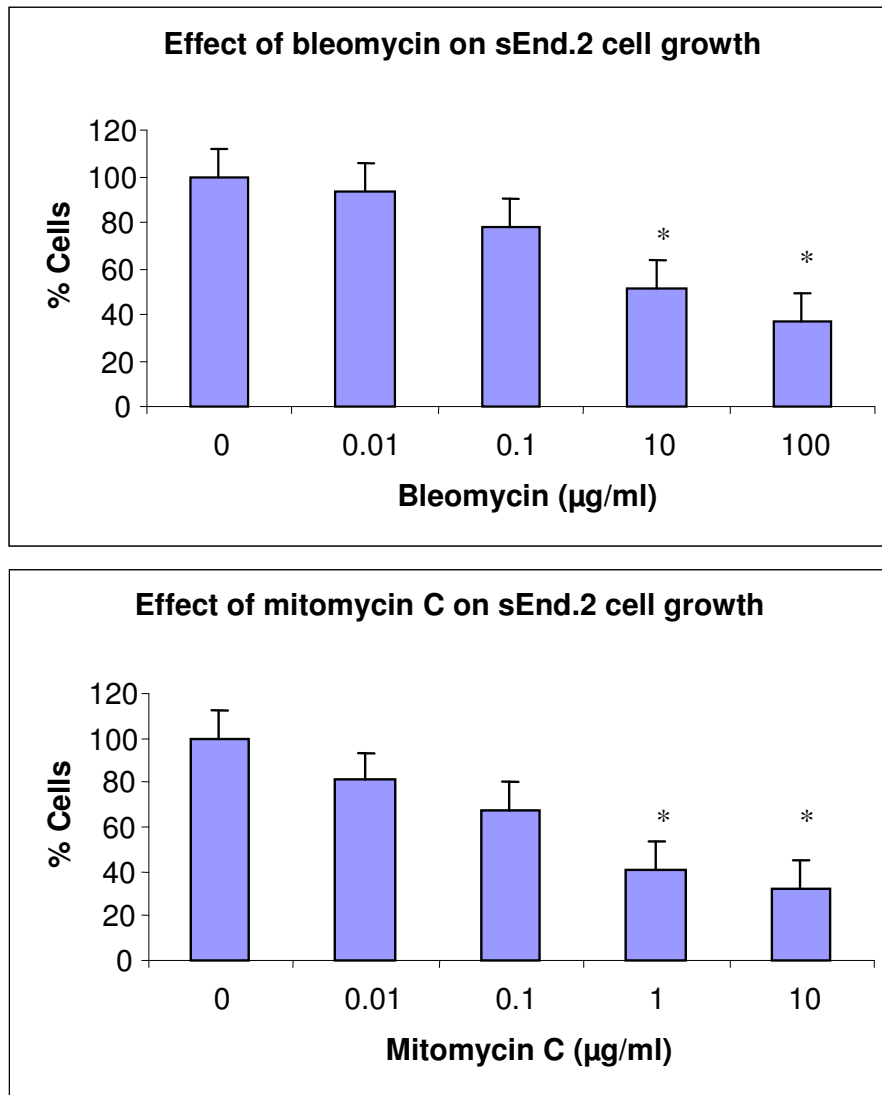


Figure 7.2. Effects of bleomycin and mitomycin C on sEnd.2 cell growth.  
\* Significant difference between the percentage of viable cells in control and drug-treated cultures ( $P < 0.05$ ).



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

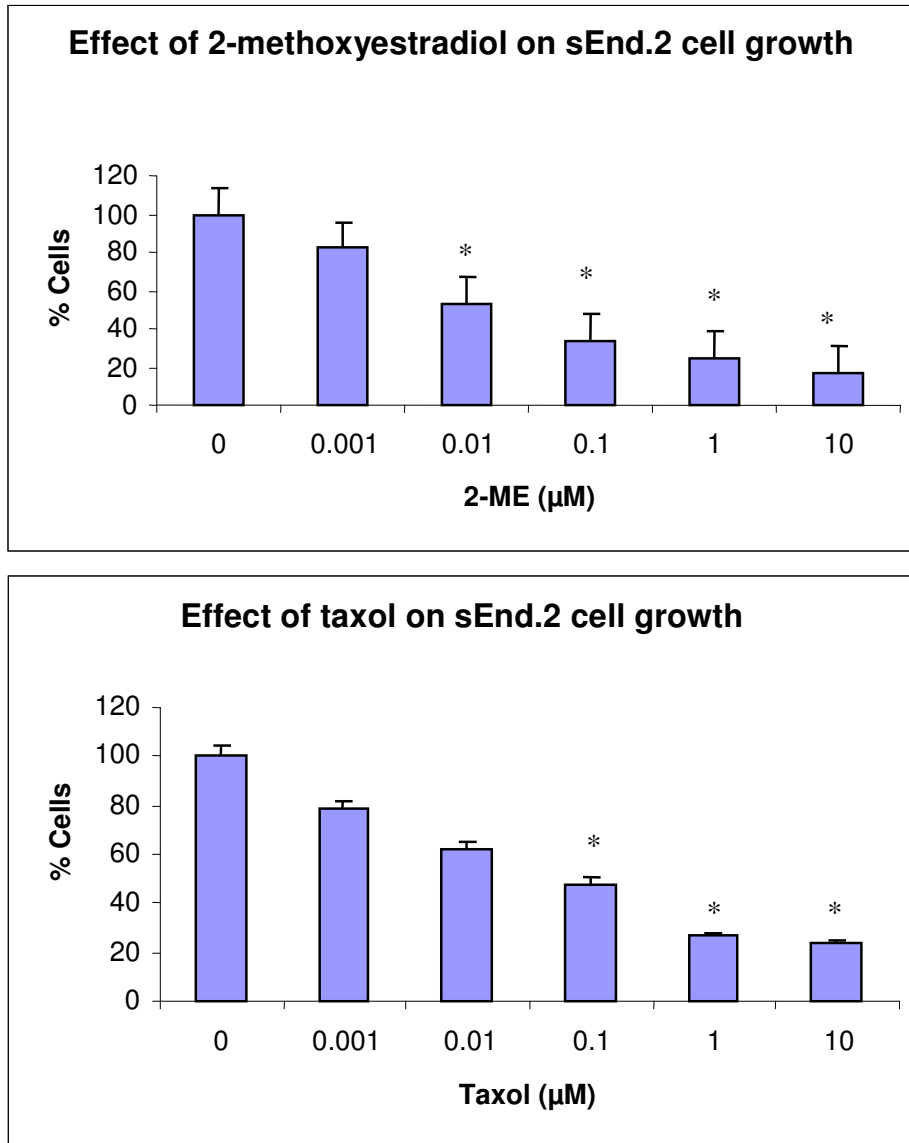


Figure 7.3. Effects of 2-Methoxyestradiol and taxol on sEnd. 3 cell growth.  
\* Significant difference between the percentage of viable cells in control and drug-treated cultures ( $P < 0.05$ ).

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

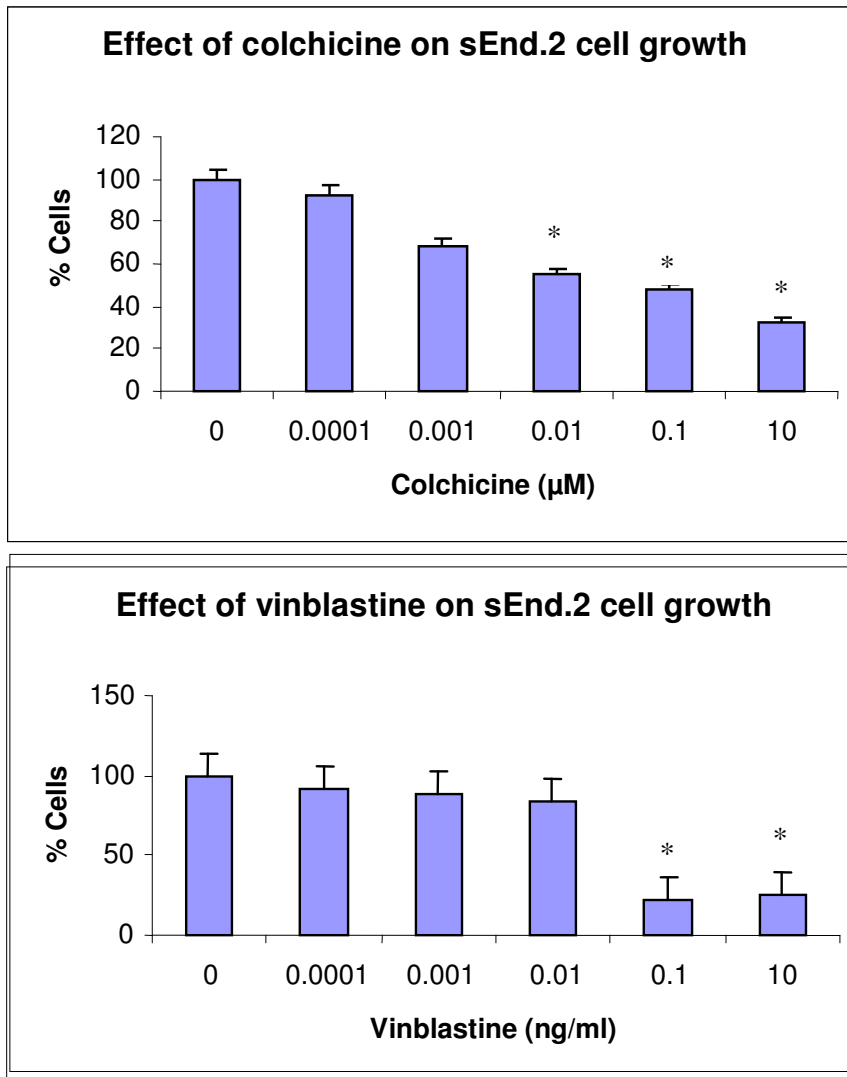


Figure 7.4. Effects of colchicine and vinblastine on sEnd.2 cell growth.  
\* Significant difference between the percentage of viable cells in control and drug-treated cultures ( $P < 0.05$ ).

Table 7.2. Concentration values of test drugs required to inhibit sEnd.2 cell growth by 50% (IC<sub>50</sub> values).

<b>Drug</b>	<b>Cell Type</b>	<b>IC<sub>50</sub></b>
Bleomycin	sEnd.2 cells	2.108 $\mu$ M (3.162 $\mu$ g/ml)
Mitomycin C	sEnd.2 cells	1.683 $\mu$ M (0.562 $\mu$ g/ml)
2-Methoxyestradiol	sEnd.2 cells	0.016 $\mu$ M
Taxol	sEnd.2 cells	0.100 $\mu$ M
Colchicine	sEnd.2 cells	0.050 $\mu$ M
Vinblastine	sEnd.2 cells	0.044 $\mu$ M (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.

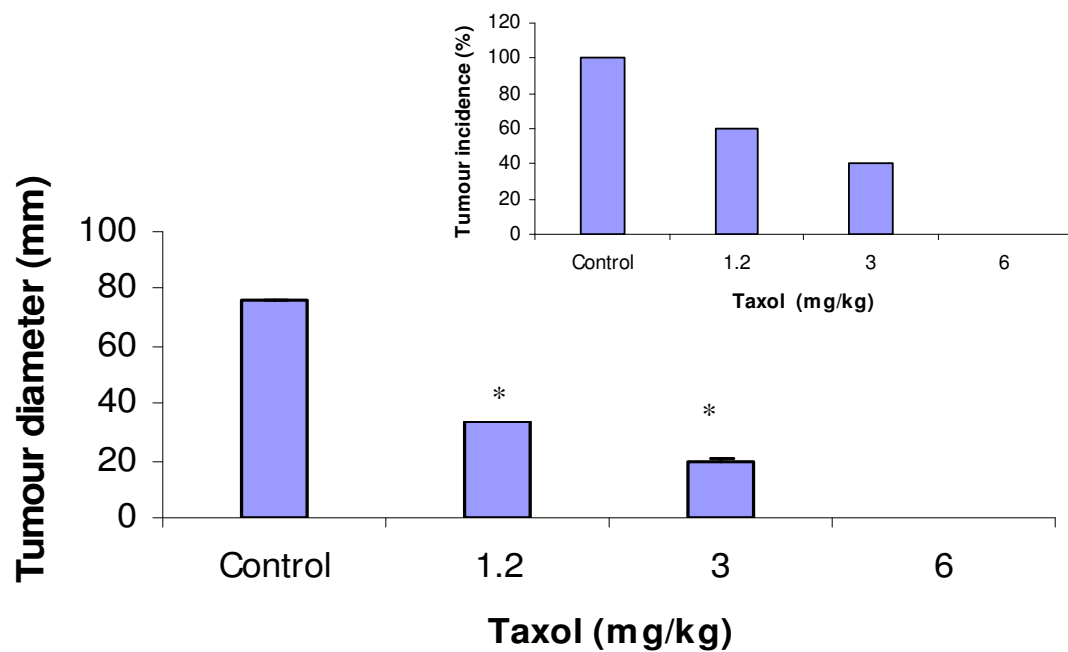


Figure 7.5. Effects of varying doses of taxol on tumour growth in mice inoculated with sEnd. 2 cells. (n = 5; 6mg/kg n = 3); \* P < 0.05.

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.

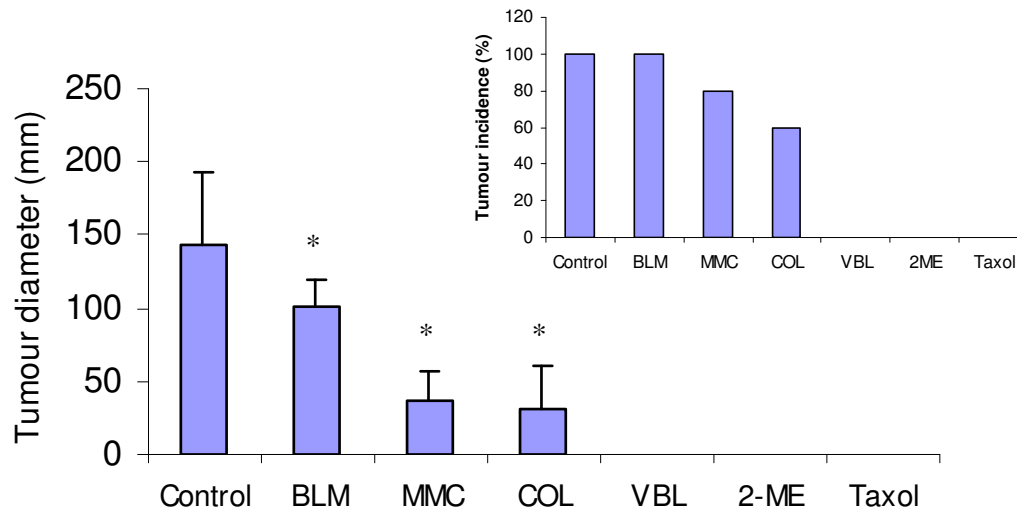


Figure 7.6. Effects of test drugs on tumour growth in mice inoculated with sEnd. 2 cells. n = 5, except for taxol. (n = 3); \* P < 0.05.

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

### 7.4.3. Histology

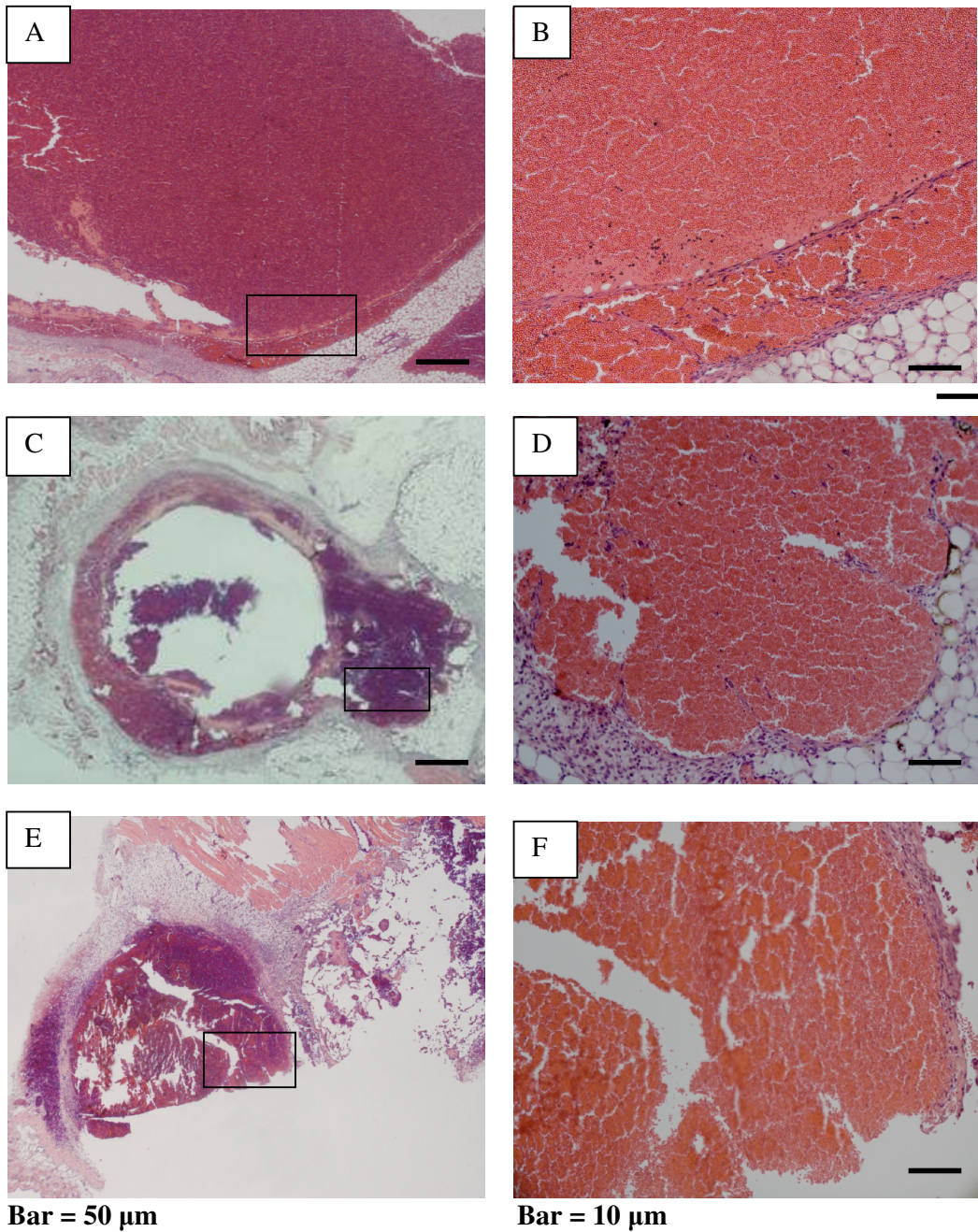


Figure 7.7. Histology of sEnd.2 cell-induced vascular tumours in mice. A and B: histology of a tissue sample from a control mouse showing with a vascular tumour composed of a layer of endothelial cells surrounding a large blood-filled lumen and inflammatory cells (stained purple). C and D: a vascular 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 (immunohistochemistry 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.<sup>15</sup>

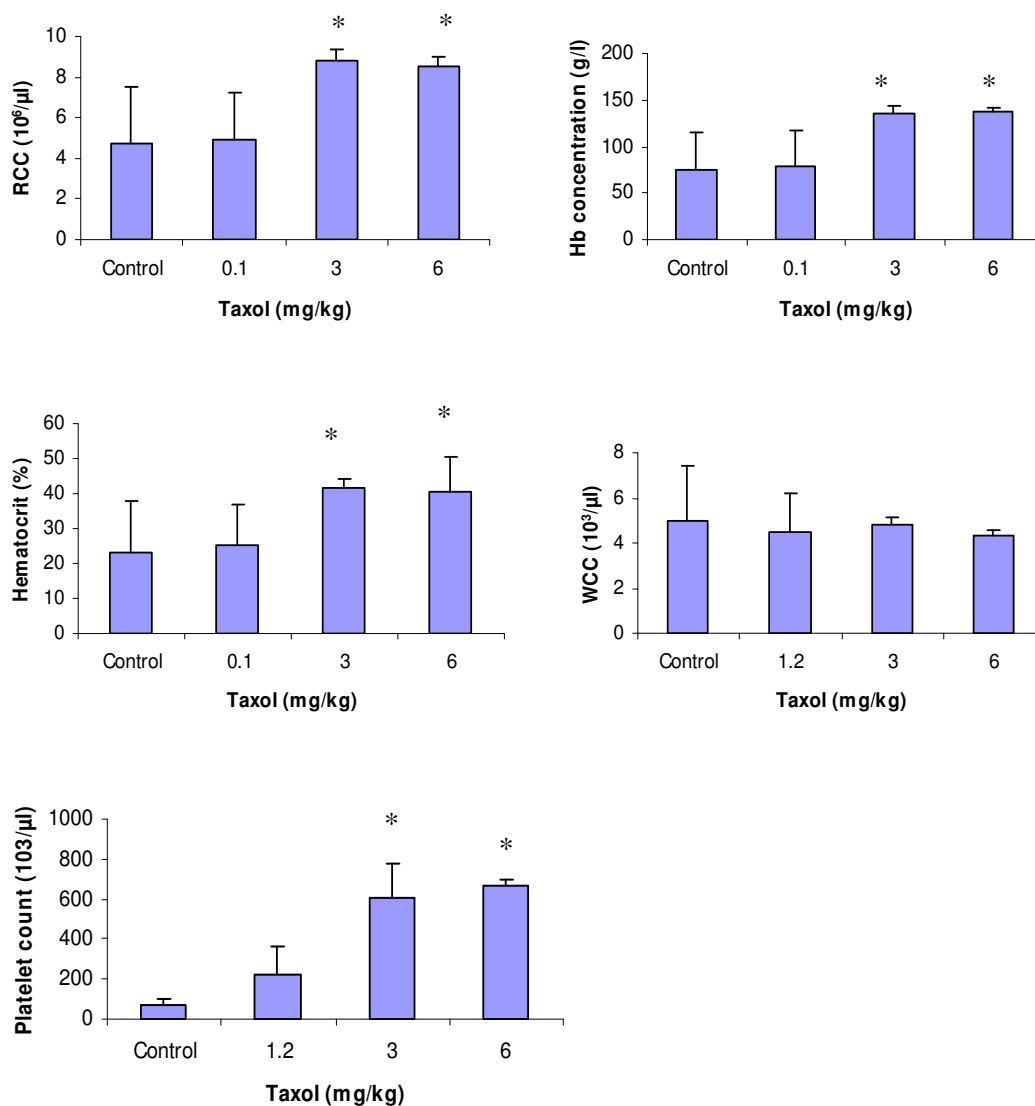


Figure 7.8. Blood parameter values (mean  $\pm$  SEM) in control and taxol-treated mice. n = 5; \* P < 0.05. RCC = red cell count; Hb = haemoglobin; WCC = white cell count.



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  $300 \times 10^3 \mu\text{l/ml}$ . The platelet count was raised in bleomycin and mitomycin C treated mice. Platelet numbers were also increased in mice treated with cytoskeletal-disrupting drugs.

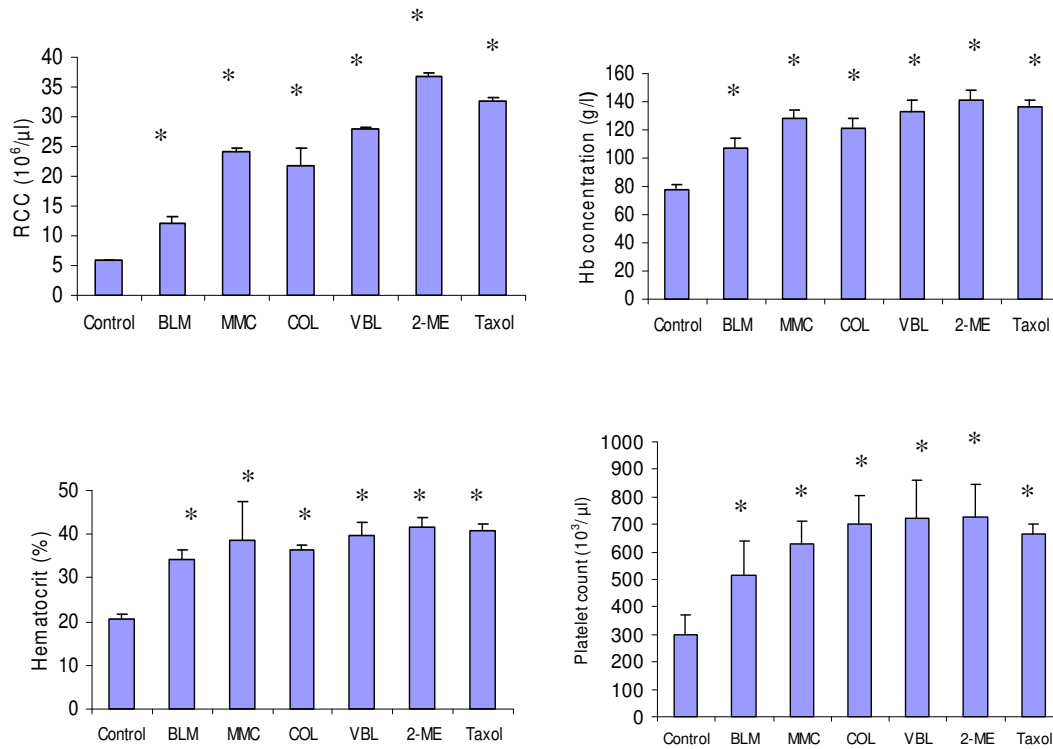


Figure 7.9. Blood parameter values (mean ± SEM) in control and drug-treated mice. n = 5, except for taxol (n = 3); \* P < 0.05. RCC = red cell count; Hb = haemoglobin.

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.<sup>15</sup> Also, the continuous presence of End. cells has been cited as being necessary to maintain the haemangioma.<sup>15</sup> On the other hand, studies have shown that injection of primary non-transformed endothelial cells induced no observable lesions.<sup>15</sup>

Histological sections from control mice showed tumours of variable size with blood filled cavities.<sup>16</sup> 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.<sup>16-18</sup> 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*.<sup>17</sup> 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:<sup>19</sup> 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.<sup>19</sup>

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  $\sim 300 \times 10^3 \mu\text{l/ml}$ , 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.<sup>20</sup> 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).<sup>21-23</sup> Previously, ultrastructural observation of KMS-associated 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.<sup>23</sup>

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.<sup>20</sup>

Thus according to Enjolras *et al.* (1997) the aim of therapeutic intervention should be ‘to increase the platelet count to a minimum acceptable level’.<sup>20</sup> 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<sub>2</sub> and B<sub>2</sub> 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 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 than 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.