CHAPTER 2

Literature Review

I. Bleomycin

2.1. Chemistry of bleomycin

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

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

2.2. Chemical Structure

A typical BLM molecule consists of 4 functional parts:

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

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

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

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

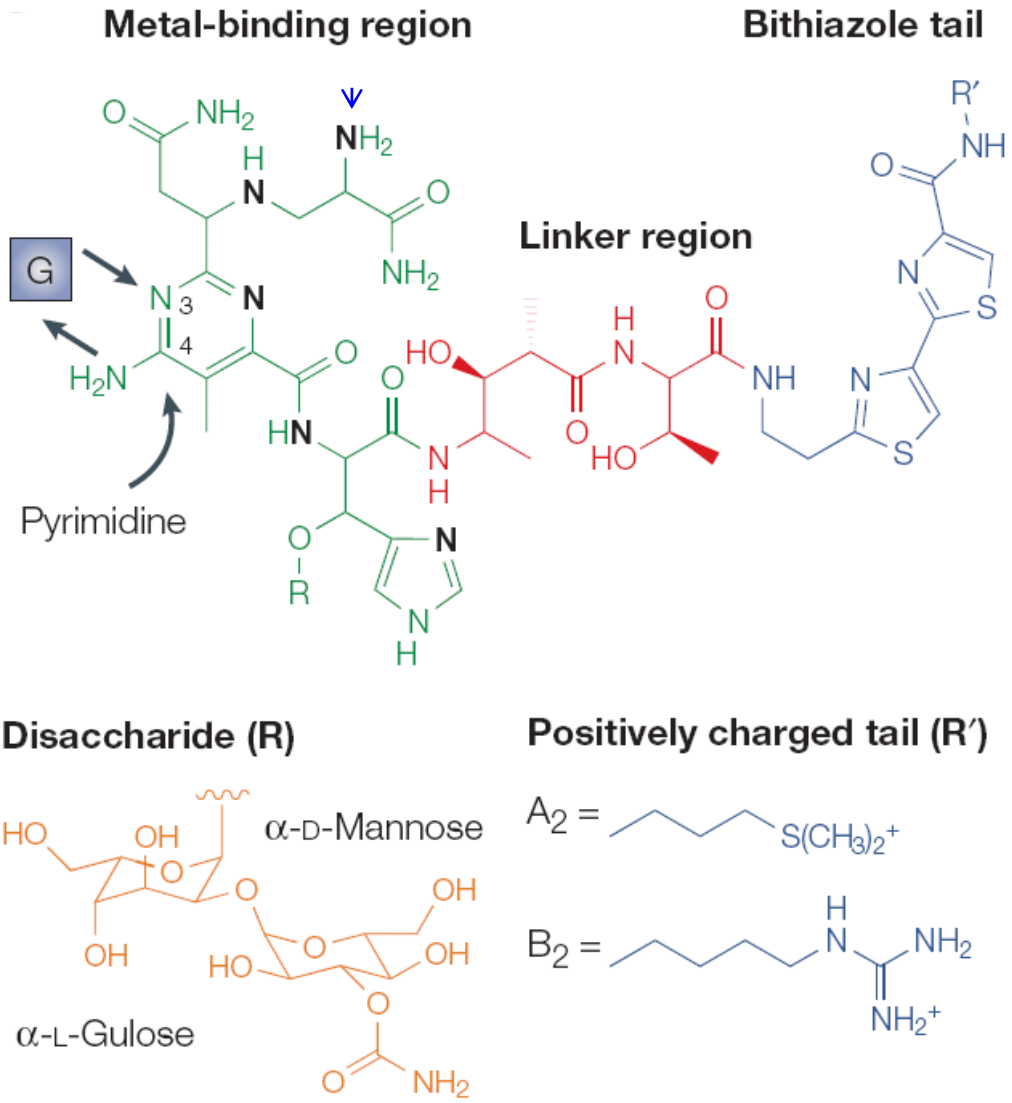

Figure 2.1. The chemical structure of bleomycin. The metal-binding domain is in green. The blue arrow points to the site of metabolic inactivation by bleomycin hydrolase. The nitrogen atoms that coordinate the metal are black. The N3- and N4- amino groups of the pyrimidine moiety of bleomycin (shown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

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

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

2.3. Metal ion Coordination

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

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

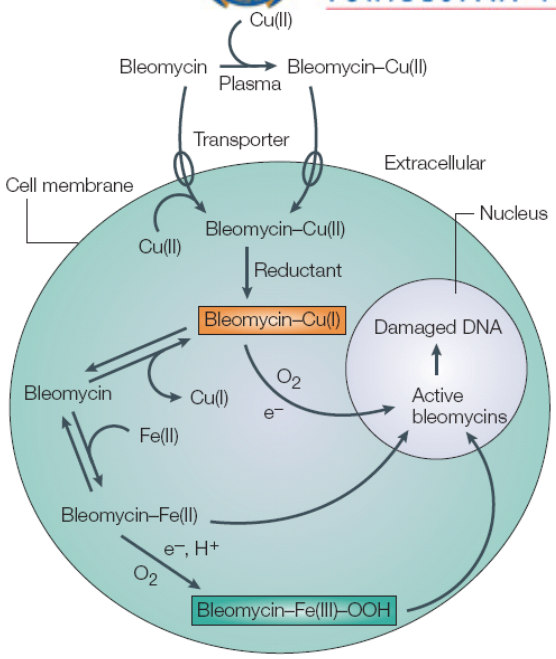

Figure 2.2. Proposed mechanism for the generation of 'activated bleomycin' *in vivo*.

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2.4. Mechanism of biological activity

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

2.4.1. Effect of bleomycin on DNA

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

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

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

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

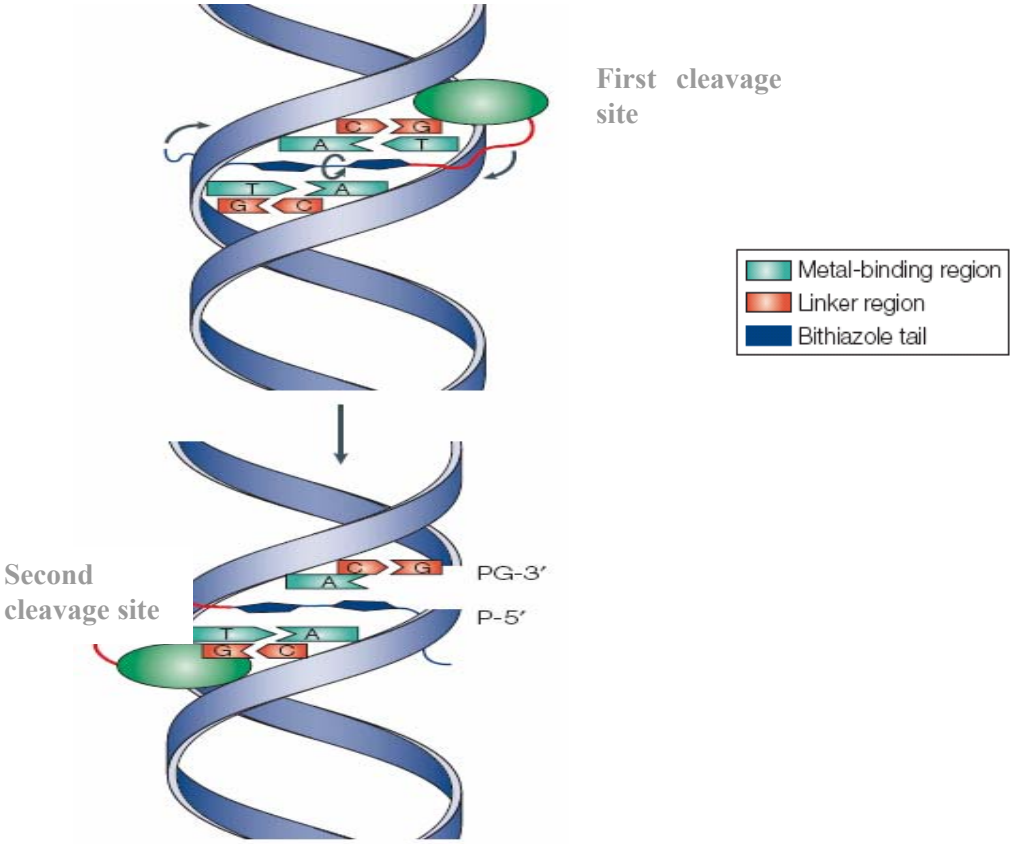

Figure 2.3. A model for dsDNA (double stranded DNA) cleavage by a single bleomycin molecule.

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

2.4.2. Effect of bleomycin on RNA

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

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

2.4.3. Effect of bleomycin on proteins

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

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

2.5. Metabolism of bleomycin

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

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

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

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

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

2.6. Side Effects

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

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

2.7. Clinical use

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

II. Haemangioma

2.8. Introduction

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

2.9. Nomenclature

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

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

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

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

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

Table 2.1: Classification of Vascular Anomalies

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

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

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

2.10. Natural History

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

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

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

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

2.11. Complications

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

2.12. Pathophysiology

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

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

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

2.13. Treatment

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

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

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

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

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

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

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

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

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

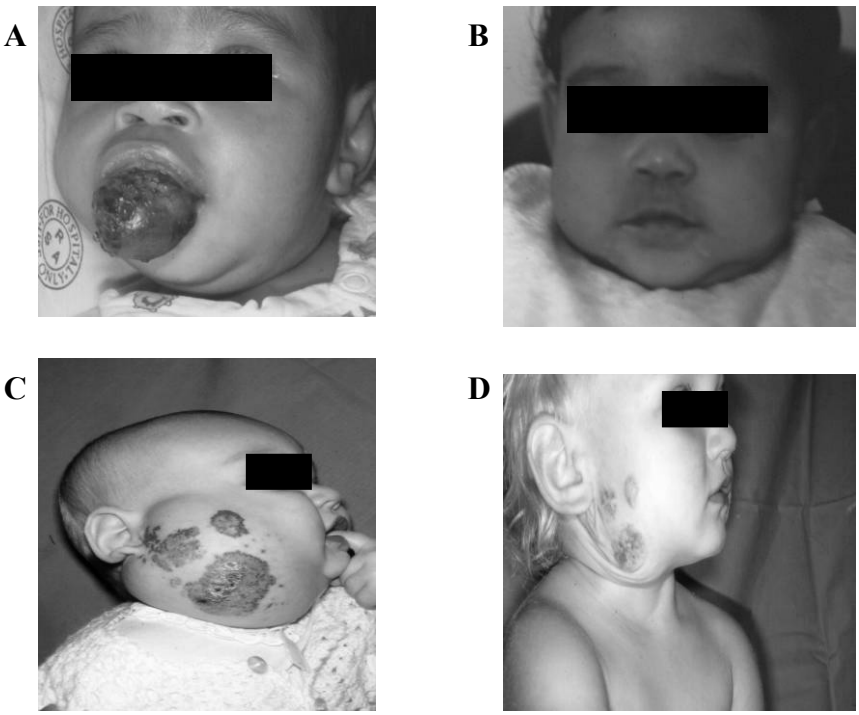
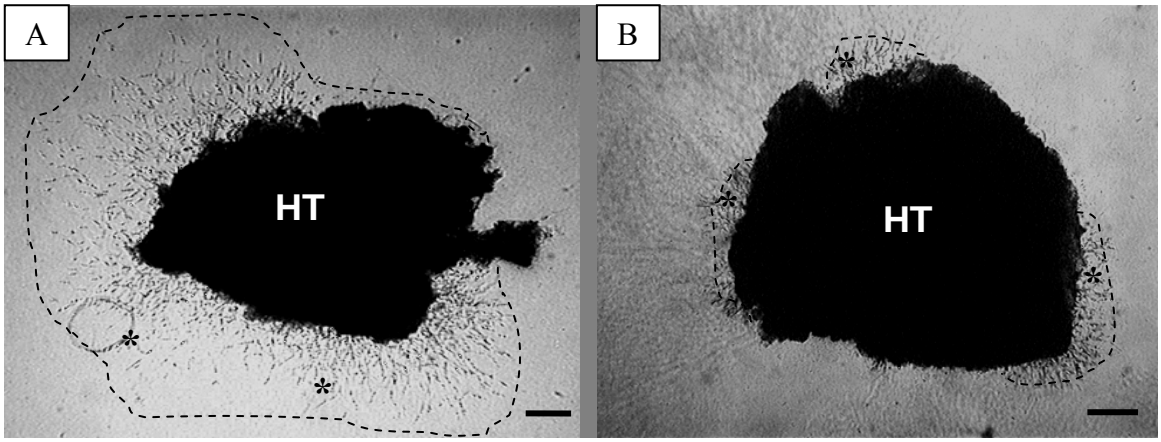


Figure 2.4. Haemangioma patients treated with intralesional bleomycin. **(A) -** a 2-month-old female infant with a histologically confirmed haemangioma originating from the superior alveolar ridge, with extra-oral protrusion of a rapidly enlarging lesion. **(B)** shows the result after 5 intralesional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

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

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

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



Bar = 250 µm

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

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

III. The Angiogenesis Concept

2.14. Introduction

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

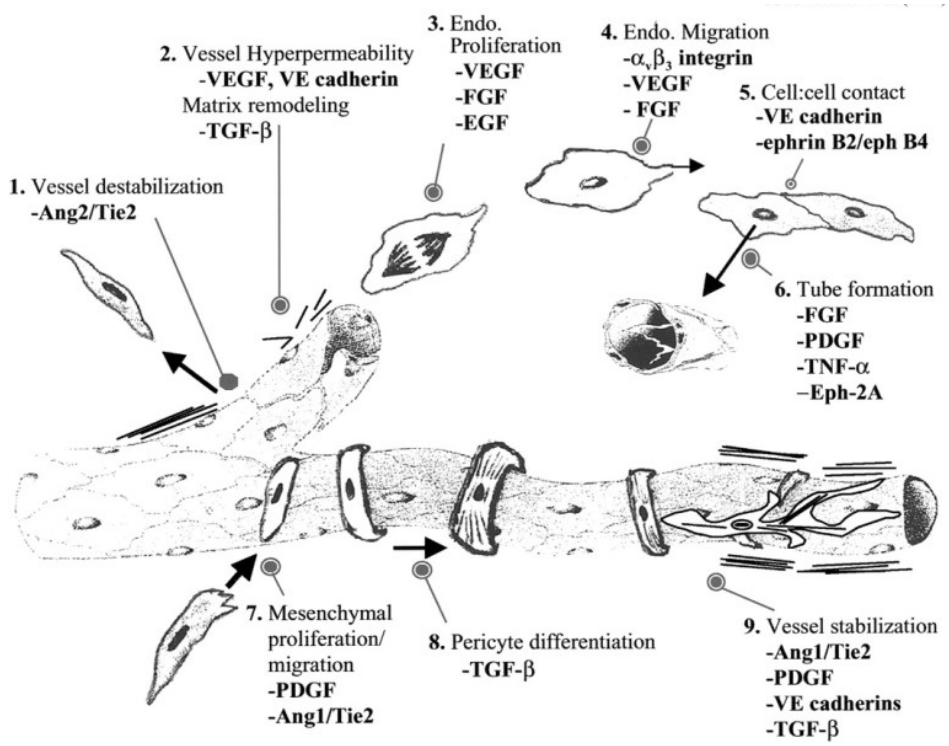


Figure 2.6. Schematic diagram illustrating the process of angiogenesis. See text for details.

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

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

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

2.15. Growth factors in angiogenesis

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

2.15.1. Vascular endothelial growth factor

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

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

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

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

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

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

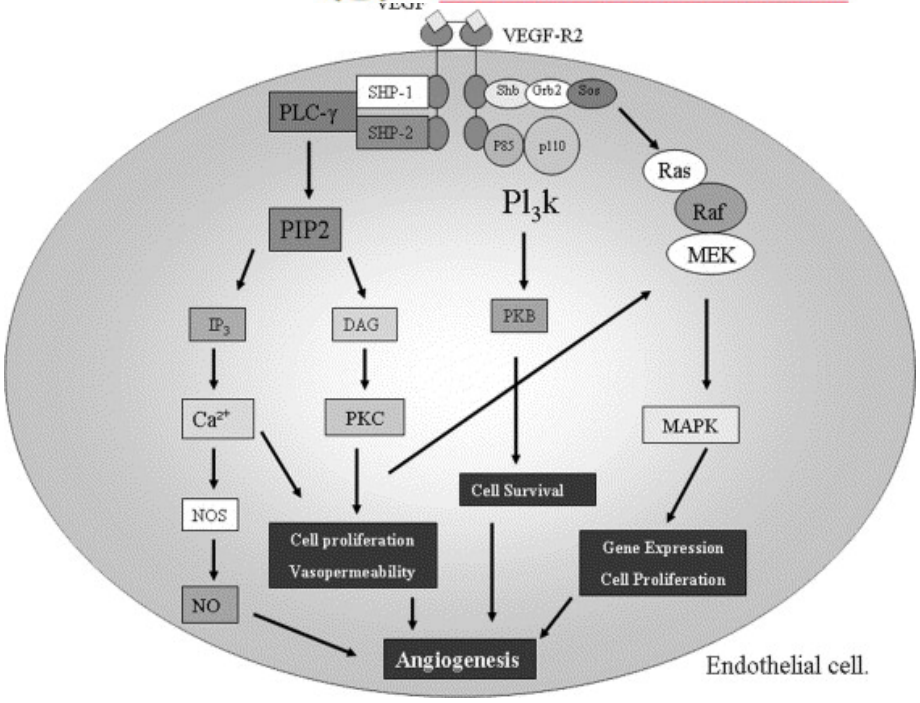

Figure 2.7. Signalling pathways of vascular endothelial growth factor.

Reproduced with permission from J Endodont 2007; 33: 524-530. Mattuella LD, Bento LW, Poli de Figueiredo JS, Nör JE, Borba de AraujoF, Fossati ACM. Vascular endothelial growth factor and its relationship with the dental pulp. [45] *Copyright (2007), with permission from Elsevier.* [76]

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

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

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

2.15.2. Basic fibroblast growth factor

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

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

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

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

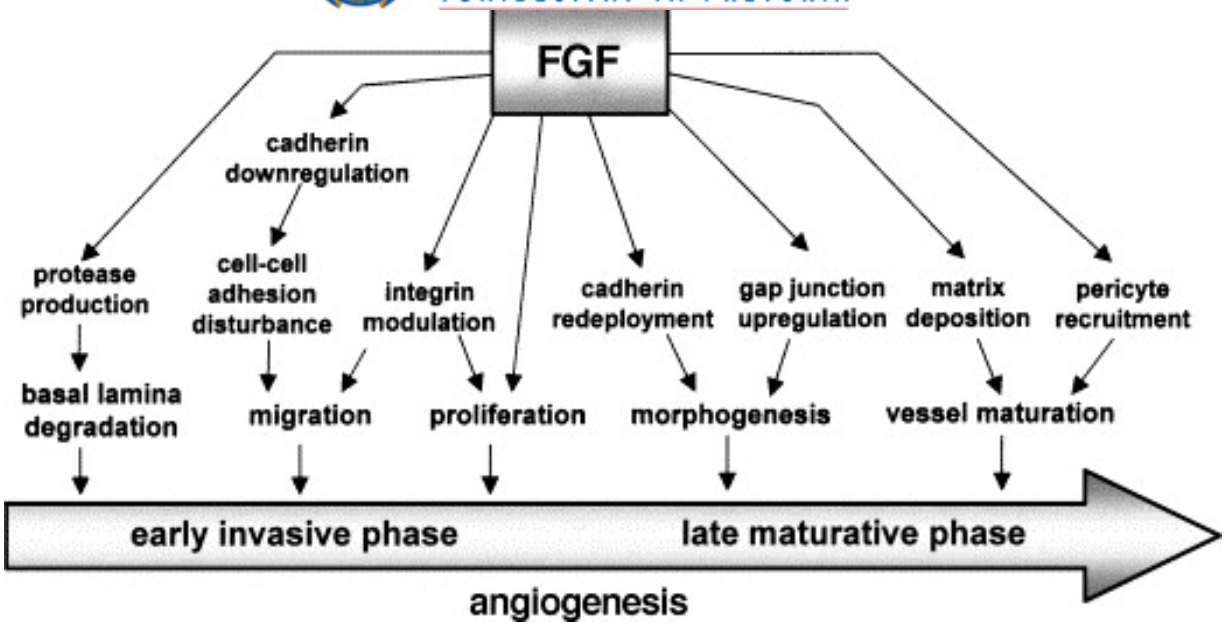

Figure 2.8. Schematic representation of the effects of bFGF in endothelial cells that contribute to the acquisition of the angiogenic phenotype *in vitro* and to neovascularization *in vivo*. *Reprinted from* Cytokine & Growth Factor Reviews 16: *159-178, Presta M, Dell'Era P, Mitola S, Moroni E, Ronca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

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

2.16. Apoptosis

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

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

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

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

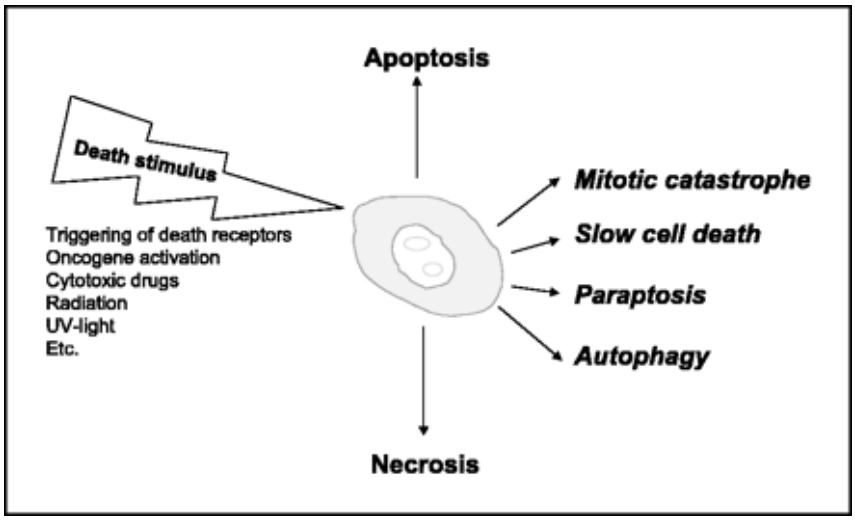

Figure 2.9. Models of cell death. See text for details.

Reprinted from Broker LE, Kruyt FAE, Giaccone G. Cell death independent of caspases: A Review. Clin Cancer Res 2005; 11: 3155-62. Permission conveyed through the Copyright Clearance Centre, Inc. [57]

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

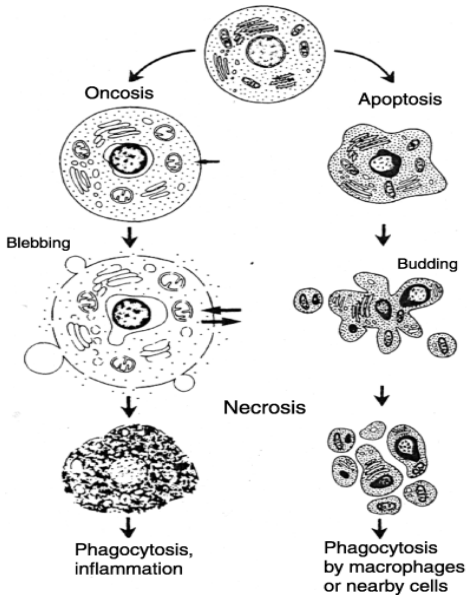

Figure 2.10. Apoptosis and oncosis.
Majno G, Joris I. Apoptosis, oncosis and necrosis. An overview of cell death. Am J Path 1995; 146:3-5. [61]

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

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

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

2.17. Mitomycin C

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

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

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

2.18. The Cell Cytoskeleton

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

2.18.1. Microfilaments

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

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

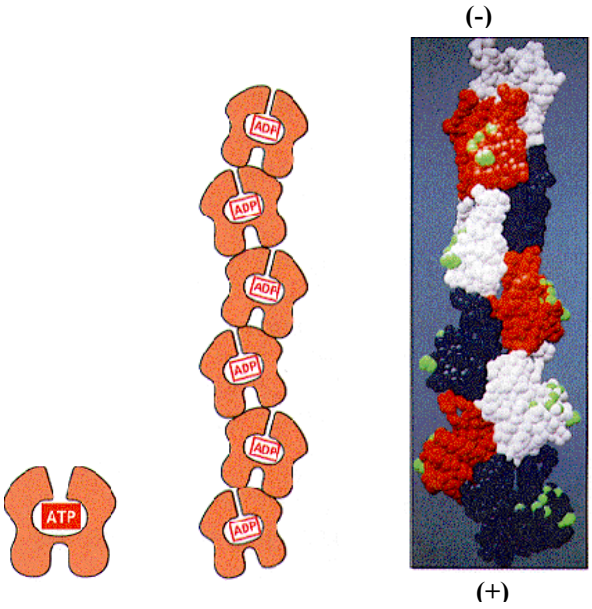

Figure 2.11. The structure of actin. (A) – an actin monomer, G-actin; (B) – a growing actin protofilament formed by multiple monomers; (C) – the actin filament, F-actin.
http:users.rcn.com/jkimball.ma.ultranet/BiologyPages/C/**Cytoskeleton**.html

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

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

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

2.18.2. Microtubules

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

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

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

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

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

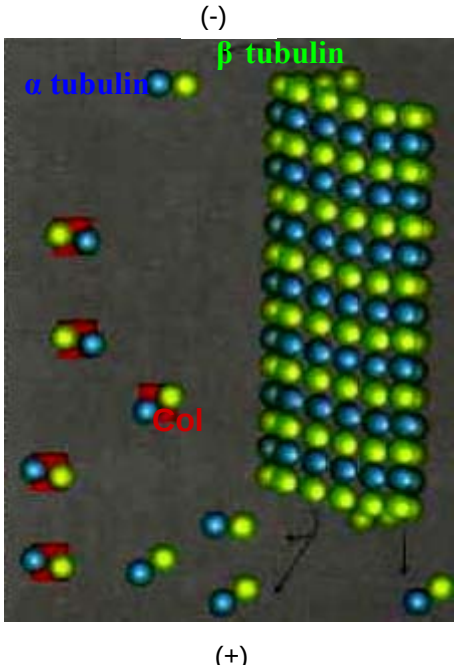

Figure 2.12. Longitudinal section through a microtubule. Colchicine (red) prevents polymerization by binding to tubulin heterodimers and thus preventing their addition to the plus end. http:users.rcn.com/jkimball.ma.ultranet/BiologyPages/C/**Cytoskeleton**.html

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

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

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

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

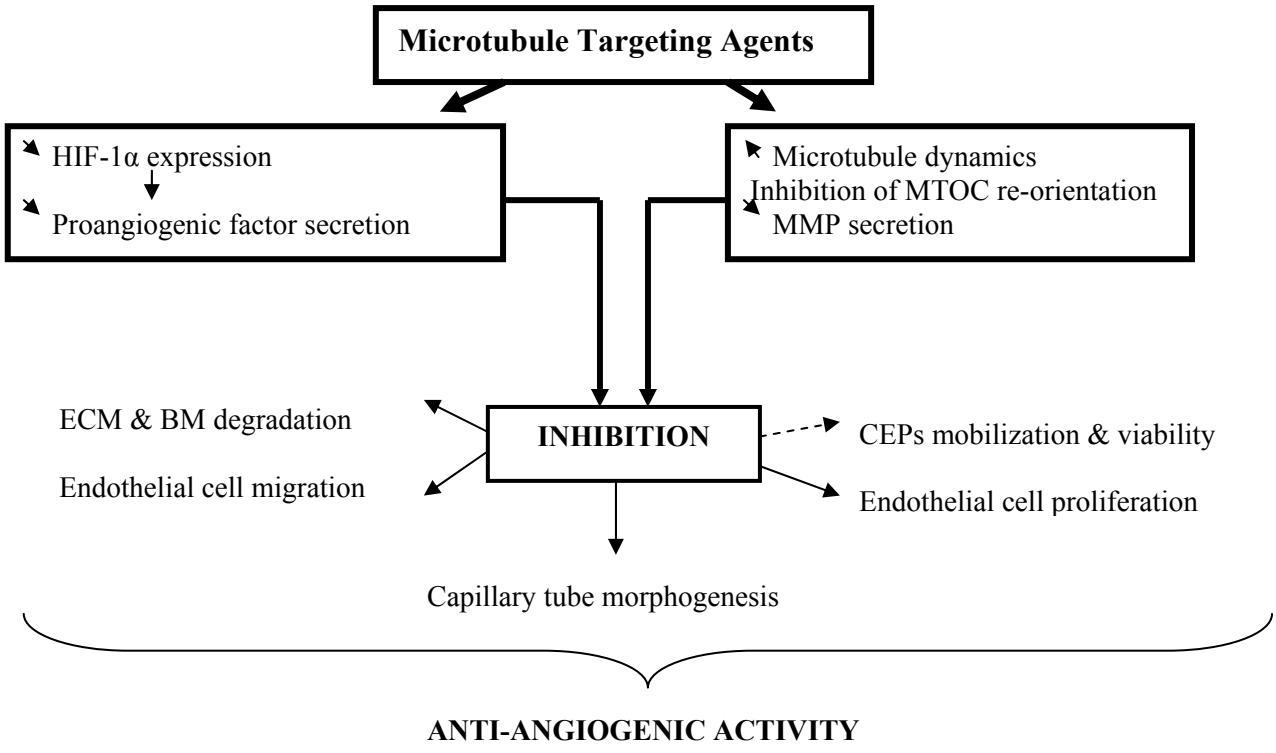

Figure 2.13. Mechanisms involved in the anti-angiogenic effects of microtubule-disrupting drugs. See text for details. HIF-1- ; MMP- matrix metalloproteinase; MTOC- microtubule organising centre; ECM-extracellular matrix; BM-basement membrane; CEPs- circulating endothelial progenitor cells.

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

2.18.3. Colchicine

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

2.18.4. 2-Methoxyestradiol

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

2.18.5. Vincristine and Vinblastine

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

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

2.18.6. Nocodazole

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

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

2.18.7. Paclitaxel

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

2.18.8. Cytochalasin D

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

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

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

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

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

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

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

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

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