The University of Hull

**Novel Anti-Endothelial Therapeutic Strategies in Malignant Melanoma –
The Metronomic Approach.**

A thesis submitted for the degree of MD
At the University of Hull.

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Abstract

Treatment strategies for advanced malignancy remain limited in their success, despite major advances in the understanding of cancer aetiology and molecular biology. The incidence of many cancers, including melanoma, continues to rise, with a huge demand for therapies even if treatment goals are purely cytostatic. One particular therapeutic strategy is the metronomic (continuous and low) dosing of conventional chemotherapy. There is evidence to suggest that tumour vasculature is the main target of this dosing schedule resulting in an overall 'non specific' anti-angiogenic effect. It is now being studied in clinical trials alone and in combination with specific anti-angiogenic agents.

This thesis had two main aims: firstly to investigate the additive or synergistic anti-endothelial effects of a number of conventional cytotoxic agents (Temozolomide, Paclitaxel, Vinorelbine, Etoposide, Carboplatin) *in vitro* given in a metronomic schedule in combination with a specific anti-angiogenic compound (Sorafenib) and a non-specific sompound (Combretastatin). The anti-proliferative, cytotoxic activities of the metronomic combinatorial schedules were assessed on microvascular endothelial cells and cancer cells using an MTT proliferation assay. Results confirmed significant ($p<0.001$) endothelial-specific anti-proliferative effects induced by cytotoxics given at metronomic doses (e.g. Temozolomide at $10\mu M$, Paclitaxel at $125\mu M$, Vinorelbine at $1nM$). These anti-endothelial effects were significantly enhanced by the addition of sorafenib ($p<0.001$) in all except for with Vinorelbine, but were not enhanced by the addition of combretastatin.

Secondly, the aim was to isolate circulating endothelial cells (CECs) and circulating endothelial progenitor cells (CEPs) in patients with advanced (stage IV) melanoma. This was a scientific clinical sub-study of an on-going clinical trial to assess the efficacy of metronomically-dosed temozolomide and estramustine. After optimisation of the isolation techniques for CECs and CEPs from whole blood samples, the metronomically-dosed participants were compared to conventionally-dosed participants. This required an appropriate MREC amendment to the already existing clinical trail. Although numbers were very small and the study was aimed at hypothesis - generation, results showed a trend towards increased CECs mid-treatment in the metronomic group (mean: 6.6 CEC/µL of whole blood pre-treatment versus 11.25 CEC/µL of whole blood mid-treatment). There was otherwise no significant difference between the two groups.

In summary, this thesis is a study of a combinatorial strategy to enhance the anti-angiogenic effects of the metronomic approach. This is a generic 'cancer treatment' approach, but is studied here more specifically in the setting of melanoma. It shows successful further development of an *in vitro* anti-endothelial assay which can potentially be used as a preclinical screening tool to determine efficacy of numerous agents and combinatorial regimens. It also demonstrates the isolation of potential biomarkers for anti-angiogenic therapy (i.e. CECs and CEPs) in a small but unique subset of patients with stage IV malignant melanoma.

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Abbreviations

A549	Human lung carcinoma cell line
AJCC	American Joint Commitee on Cancer
ALL	Acute Lymphoblastic Leukaemia
BSA	Bovine serum albumin
CA4P	Combretastatin – drug
CEC	Circulating endothelial cells
CEP	Circulating endothelial progenitor cells
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DTIC	DTIC-Dome / Dacarbazine
EDTA	Ethylene Diamine Tetra-acetic Acid
EPC	Endothelial progenitor cell
FACS	Fluorescence-activated cell sorter
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein Isothiocyanate
GDP	Guanosine diphosphate
HIF-1	Hypoxia-inducible factor-1
HSC	Haematopoietic stem cell
HR	Hazard ratio
HUVEC	Human umbilical vein endothelial cell
IFNα	Interferon alpha
IL	Interleukin
IV	Intravenous
LDH	Lactate dehydrogenase
MACS	Magnetic-activated cell separation
MAPK	Mitogen-activated protein kinase
MCF-7	Human breast carcinoma cell line
MMP	Metalloproteinase
MTD	Maximum tolerated dose
MTT	3-(4,5-Dimethlthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
MVD	Microvascular Density
MVEC	Microvascular endothelial cells

NCCN	National Comprehensive Cancer Networks
OBD	Optimal biological dose
ORR	Objective response rate
OS	Overall survival
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD-ECGF	Platelet-derived endothelial cell growth factor
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PFS	Progression free survival
RCT	Randomised Controlled Trial
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SK-MEL2	Human melanoma cell line
TAF	Tumour angiogenesis factor
TNF	Tumour necrosis factor
TSP-1	Thrombospondin-1
TTP	Time to progression
UICC	International Union Against Cancer
VEGF	Vascular endothelial growth factor
VEGF-R	Vascular endothelial growth factor receptor

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I would like to dedicate this thesis to my parents. To my mum for her support, continual positivity, and love. To my dad, for his inspirational passion towards science and his dedication to achieve the best possible outcome from anything that one is striving towards – something I shall always try to live by.

Publications and Presentations in support of this thesis

Papers

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Little S, Murray A, Stanley P, Maraveyas A, Cawkwell L. In-vitro chemosensitivity testing to determine synergistic activity with combretastatin A4 phosphate in a microvascular endothelial cell model. *Submitted 2011.*

Presentations

Murray A, Little S, Sreetharan V, Cawkwell L, Maraveyas A. In vitro synergy between continuous low dose Temozolomide and Sorafenib. *Hull York Medical School Research Conference, York, March 2007.*

Little S, Murray A, Sreetharan V, Cawkwell L, Maraveyas A. Defining compound synergy in angiomics. *9th International Symposium of Anti-Angiogenic agents, San Diego, California, US, February 2007.*

Murray A, Ashraff S, Stanley P, Gilbert P. *ABC* of molecular biology for the plastic surgeon – *A*ngiogenesis, *B*iological mechanisms and *C*ancer. *Winter Scientific Meeting for the British Association of Plastics, Reconstructive and Aesthetic Surgeons, Royal College of Surgeons, London, December 2007.*

Chapter 1

Angiogenesis, Melanoma and Metronomic Chemotherapy

1.1   Introduction

Cancer remains one of the leading causes of death in the Western World. It is a major cause of morbidity in the United Kingdom, and each year more than a quarter of a million people are newly diagnosed with cancer. Cancer is primarily a disease of older people; therefore a person's risk of developing cancer is dependent on age. Overall, it is estimated that more than 1 in 3 people will develop some form of cancer during their lifetime, compared to an estimated risk of 1 in 27 for people aged up to 50 year. Less than 1% of all cases occur in children, aged 0 to 14 years (CancerResearchUK 2011).

Despite an overall decreasing mortality rate for malignant neoplasms as a whole, the incidence of most cancers continues to rise. The most rapidly increasing incidence rates include a quadruple rise in incidence rates of malignant melanoma over the last thirty years, with around 11,760 cases diagnosed in 2008. A similarly significant increase in the incidences of liver cancer, non-Hodgkin lymphoma and prostate cancer are also reported. Along with this, an increase in mortality rates has been associated with such malignancies. In the 60-79 year old male group, a 790% increase in melanoma incidence was noted between 1975 and 2008 (CancerResearchUK 2011).

Although our understanding of cancer aetiology, molecular biology and treatment

strategies has without doubt advanced dramatically in the last few decades, treatment strategies for advanced malignancy remain limited in their success. The compilation of different chemotherapeutic regimens and other cancer therapies, such as immunotherapy, continue to often be empirically devised or based on lower level evidence (Balch C *et al* 1997; Huber P *et al* 2005; CancerResearchUK 2011).

The era of cancer chemotherapy began in the 1940s with the first use of nitrogen mustards and folic acid antagonist drugs. Cancer drug development has exploded since then into a huge industry, and now targeted therapies have started to revolutionise the methods of cancer treatment. However, many of the early principles still apply. When Louis Goodman and Alfred Gilman – both pharmacologists - were recruited by the United States Department of Defense to investigate potential therapeutic applications of chemical warfare agents, they discovered at autopsy that profound lymphoid and myeloid suppression had occurred in people exposed to mustard gas. They applied this finding to the treatment of lymphoma in mice and later in humans, and found there to be a dramatic reduction in patients' tumour mass as a result of the agent (Goodman L *et al* 1946; Gilman A *et al* 1963) .

Shortly after World War II, a second approach to cancer therapy began. Sidney Farber, a pathologist at Harvard Medical School, studied the effects of folic acid on leukaemia patients. Folic acid, a crucial vitamin for DNA metabolism, was found to stimulate the proliferation of acute lymphoblastic leukaemia (ALL) in children. Following this observation, Farber and colleagues synthesised folate analogues e.g. aminopterin and then amethopterin (now methotrexate) which acted antagonistically to folic acid, and were found to induce remission in children with ALL. Farber's

report at the time was met with ridicule and it was felt that the children should be left to die in peace (Farber S *et al* 1948).

Following the work of Farber, the rationale behind his drug design led to an era of anti-metabolite development, whereby small changes were made to molecules required for cell division. This led to the discovery of 6-mercaptopurine, which was subsequently shown to be a highly active anti-leukaemic drug. Around the same time, the Eli Lilly natural products group found that alkaloids of the Madagascar periwinkle (Vinca rosea) were able to block proliferation of tumour cells, later found to be via inhibition of microtubule polymerisation, and therefore cell division (Johnson I *et al* 1963).

As a result of these discoveries, the United States Congress created a National Cancer Chemotherapy Service Center (NCCSC) in 1955, in order to promote drug discovery for cancer. The NCCSC developed crucial methodologies and tools, such as cell lines and animal models, for chemotherapeutic development. This early revolution in cancer therapy was therefore largely a North American experience, fuelled by an optimistic United States Federal government, following the 'big-idea' philosophy that they also applied to the Apollo Program (Kennedy JF 1961). It was only later that the pharmaceutical industry became heavily involved, realising the potential for financial gain.

In 1965, a major break-through in cancer therapy occurred when James Holland and his colleagues hypothesised that cancer chemotherapy, in particular that for leukaemia, should follow a strategy of antibiotic therapy for tuberculosis with combinations of drugs (Holland J 1965). This was thought to reduce the likelihood of

tumour resistance to develop. The group devised a regimen of methotrexate (anti-folate), vincristine (Vinca alkaloid), 6-mercaptopurine and prednisolone – together referred to as POMP regimen – and were able to induce long-term remission in children with ALL. ALL became largely a curable disease, as it is today. This approach was later applied to adult lymphoma, and currently all curative cancer chemotherapy uses a multiple drug paradigm (Yates J *et al* 1974).

Next came the concept of adjuvant therapy, whereby tumour burden was first reduced by surgery, then chemotherapy would follow, in an attempt to clear any remaining malignant cells. This was shown to improve survival in some patient groups, and coincided with further drug design and development. The classes of agents that then came into the fore-ground included the taxanes – acting on the cellular micro-tubules, the vinca alkaloids and the camptothecins – inhibitors of topoisomerase I, an essential enzyme for the structuring of DNA (Wall M *et al* 1966). The taxanes act on the microtubules by stabilizing GDP-bound tubulin in the microtubule. This results in a 'freeze' of mitosis. Thus the taxanes are mitotic inhibitors. In contrast the vinca alkaloids destroy mitotic spindles, and therefore the 2 classes of agents have been widely termed spindle poisons. Some of these agents, along with the topoisomerase inhibitors, subsequently gained FDA approval for the treatment of varying cancers including ovarian (Paclitaxel) and colon (Irinotecan) cancer.

Other classes of agents were also being developed and were shown to have some success in treating various malignancies, including platinum-based agents (e.g.Cisplatin) in testicular cancer. Interestingly, many derivatives from these different classes of agents were found to have different side-effects and often non-

therapeutic overlapping properties e.g. cisplatin and oxaliplatin – where oxaliplatin can work in cases of cisplatin resistance when used as part of a multi-targeted approach (Stordal B *et al* 2007).

Throughout this period of surge in drug development, chemotherapeutic regimens were based on the 'maximum tolerated dose' (MTD) paradigm (Skipper HE *et al* 1970). Phase I trials aim to determine the optimal dose of a new compound for subsequent testing in phase II trials. With cytotoxic agents, this dose has traditionally corresponded to the highest dose associated with an acceptable level of toxicitiy. This is based on the assumption that the higher the dose, the greater the likelihood of drug efficacy. In addition to the relationship between dose and anti-tumour response, cytotoxic agents also exhibit a dose-toxicity relationship. Therefore, dose-related toxicity is generally and traditionally regarded as a surrogate marker for efficacy (Skipper HE *et al* 1964). Clinical investigators soon realised that the ability to manage these dose-related toxicities was crucial to the success of cancer chemotherapy. Support on MTD regimens often involves platelet and red-cell transfusions, and antibiotic cover to treat any infective complications resulting from the myelotoxic effects of these cell poisons. Most of the traditional chemotherapeutic agents also induce nausea and vomiting - often unbearable to patients - although anti-emetic agents e.g. ondansetron, have reduced the severity of these side effects.   A low percentage of 'toxic' deaths (usually less than 1.5%) is well described and has become 'acceptable'.

 Although transient disease regressions in advanced dsease are not uncommon with the MTD approach, eventual drug resistance is almost always acquired by the tumour

phenotype (Longley DB and Johnston PG 2005). Furthermore, the potentially severe and systemic adverse effects of MTD cytotoxic therapy can lead to a reduced quality of life and even death, in patients in whom prognosis is poor and treatment is palliative from the outset. There was therefore felt to be a huge need to develop alternative and more successful strategies for the treatment of all cancers, particularly for those with a rising incidence and where treatment options so far have had little impact on overall survival e.g. malignant melanoma. Achieving this in a more targeted sense with commensurate reduction of toxicity would be a welcome development, and is currently a rapidly expanding field in cancer treatment and research.

It is still early days in understanding the machinery of the cancer cell and its genetic make-up; however insight into some of the mechanisms underlying cancer growth, invasion and metastases has been paramount in the development of targeted cancer therapy. Prior to this new approach, chemotherapeutic agents had been discovered either by chance, or by inhibiting metabolic pathways crucial to the division of all cells, but none were yet specific to the cancer cell. The exception to this was the development of 5FU by Robert Duschinsky, a German scientist, in the late 1950's. His scientific goal was to develop a drug that demonstrated specific uracil antagonism, since uracil is a normal component of RNA, exploiting the rapid division of cancer cells (Heidelberger C *et al* 1957). This agent has subsequently gone on to be the key agent used in many chemotherapy regimens used in cancers such as colon, pancreatic and breast. **Figure 1.1** summarises the sites of action of cytotoxic agents on dividing cells.

Figure 1.1: Cell Cycle Summary and Site of Action of Cytotoxic Agents on Dividing Cells
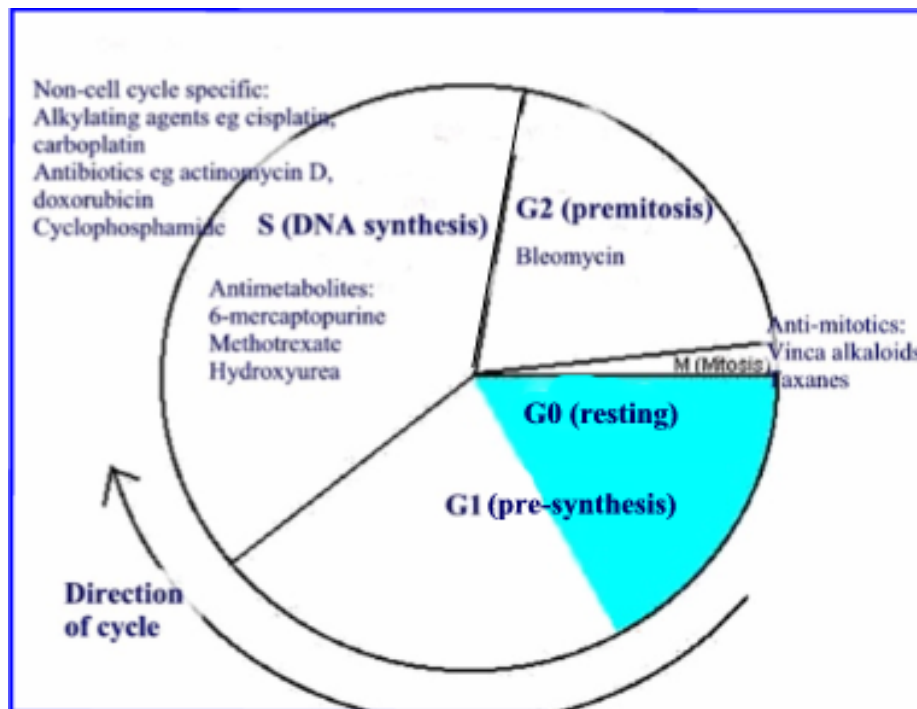
One of the most exciting alternative treatment strategies, which has received much interest over the last decade, is that of inhibiting tumour blood vessel growth, otherwise termed anti-angiogenesis strategies. Angiogenesis is the process by which tumours acquire a blood supply for continued growth and survival, and therefore represents a potentially effective cancer target. The list of agents that seem to have an anti-angiogenic approach is expanding exponentially (Quesada A *et al* 2006; Albini A *et al* 2007; Ribatti D *et al* 2010) and features not only small-molecules which target specific signalling pathways, but also some of the conventional cytotoxic agents. The relatively novel idea that traditional cytotoxic agents may in fact induce an anti-angiogenic effect when given continuously at low-doses has been termed the 'metronomic approach', and will be discussed in detail later in this chapter (section 1.3). This may potentially play a major part in the future of modern cancer treatment. The focus had been, until relatively recently, on the development of further conventional chemotherapeutic agents. These agents however, have fallen short of the high expectations of curing the most common of cancers, particularly once these cancers have metastasised. A new era of targeted cancer therapy is now well underway and much overdue. The trend is shifting towards the development and use of small molecular inhibitors of aberrant proteins and immunotherapy, in an attempt to develop more tumour specific, patient-friendly and effective regimens.

This introductory chapter discusses the background, evidence and justification for adopting targeted cancer strategies, in particular, those which target angiogenesis and their use in the treatment of malignant melanoma.

1.2   Angiogenesis

1.2.1   Definition

Angiogenesis is the formation of blood vessels and vascular networks from pre-existing vasculature. It is an important natural process occurring within the body, both in health and disease (Carmeliet P 2003). In health, this process helps to heal wounds and restore blood flow after injury or insult. In females, angiogenesis occurs during the monthly cycle to help rebuild the uterine wall, and during pregnancy, it occurs in the construction of the placental circulation. In such healthy situations, there is a fine balance between stimulation and inhibition, and in general, the angiogenesis switch is off (Hanahan D and Folkman J 1996).

The primary formation of blood vessels is termed vasculogenesis, and occurs via differentiating angioblastic cells (Risau W 1997). In the embryo, this is via mesodermal angioblastic cells which go on to form a capillary plexus, whereas in the adult, it is via recruitment of the bone marrow angioblastic cells (Augustin HG 2001). The process of angiogenesis is therefore secondary to vasculogenesis (**Figure 1.2**) as it occurs from pre-existing vessels. It can be 'sprouting', involving anastomoses formation, or 'non-sprouting', which involves intussusceptive microvascular growth resulting in a tissue pillar or the fold-like splitting of a vessel. Both these processes contribute to the formation of a complex and vascular network, which can allow directional blood flow. Biomechanical and cellular factors contribute to the shaping of vascular structures, and finally micro-environmental factors e.g. the extracellular matrix, regulates the organ-specific differentiation of the neovascular tree (Zhu W *et al* 2000).

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Figure 1.2: Development of vascular systems

During vasculogenesis, endothelial progenitor cells give rise to primitive arteriovenous labyrinths; in angiogenesis, this is expanded by additional pericytes (PCs) and smooth muscle cells (SMCs) to form organised vascular networks.
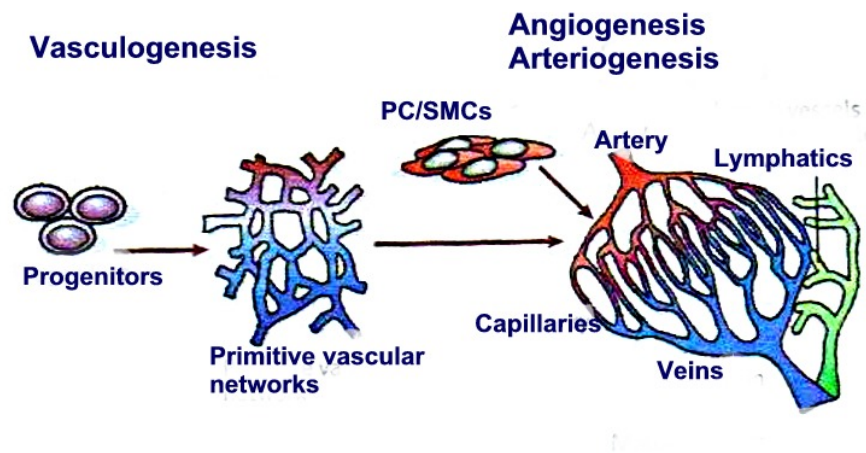
Diagram adapted from Carmeliet 2003.

Readaptation of existing mature vasculature is termed 'remodelling', and this may occur in response to acute or chronic stimuli e.g. hypertensive biomechanical forces. An existing vascular bed that contributes to tumour vascularisation, may well undergo similar remodelling processes, and this is termed 'co-option' (Augustin HG 2001).

It has become apparent that angiogenesis is a complex process and our understanding of all the pathways involved is still far from complete. Its relevance to tumour growth, however, is unquestionable and this chapter will explain the significance of this.

1.2.2   Background to tumour neovascularisation

The field of angiogenesis has grown exponentially over the last 30 years, but it by no means is a 'new' area of study. Dating back as far as 5AD, Aulus Cornelius Celsus pronounced the signs of inflammation to be calor (heat), rubor (red), tumor (swelling) and dolor (pain) (Celsus AC 1935). At the time, these clinical signs were associated with an excess of blood. However, the actual circulation of blood and the concept of microvasculature were not elucidated until the work of William Harvey (1578-1657), who never ventured to ancient Rome or Greece, and hence the link was not made.

The French surgeon, Ambroise Pare (1510-1590), went on to discuss the use of cautery in the management of cancer, noting the importance of targeting tumour blood supply. Pare explains that "…the fashion of a Crab doth represent the horrid forme of that ulcer" (Pare A 1634), comparing the projecting legs of the crab to the abnormal tumour vasculature.

Later, in the Eighteenth century, John Hunter – Scottish surgeon and scientist - noted that vessels have an immense 'power of perfecting themselves' and that 'it is they which perform the operation of restoration on the other parts' (Hunter J 1728-1793). As such, Hunter recognised the significance of angiogenesis, and also went on to unravel one of the major anatomical mysteries of his time – the lymphatic system.

Many more acclaimed scientists and surgeons continued to observe the phenomenon of tumour growth and their vasculature. In October 1907, Professor Golmann of Freidurg gave a lecture at the Royal Society of Medicine in London, outlining the chaotic irregularity of tumour vasculature, and the theory that tumours exerted an angiogenic influence on surrounding tissue (Goldman E 1907).

In 1945, Algire and Chalkley studied the vascular reaction of host tissue to tumour growth. They compared the vascular growth in wounds, in implanted benign tissue and in implanted sarcomas and carcinomas. New vessel growth, quantified microscopically, was seen at three days in the cancers and six days in benign lesions and wounds. They concluded that the tumours produced a specific substance which induces vessel growth (Algire GH *et al* 1945). Following on from this, Pietro Rondoni, Professor of Pathology at the Univesity of Milan (1946) wrote that " a tumour acts both angioplastically and angiotactically" (Rondoni P 1946) and hypothesised that a cancer induces formation of new blood vessels from those that surround it (Ribatti D *et al* 2001).

The search for a solution continued, and by 1971, Judah Folkman presented the theory that tumours were entirely dependent upon vessel formation for growth,

progression and metastasis (Folkman J 1971). His work, using an isolated organ perfusion system, demonstrated the reliance of tumour survival on endothelial recruitment. This placed angiogenesis at the centre of tumour biology. Following on from this, Gimbrone et al investigated the existence of a fundamental relationship between angiogenesis and the ability of a tumour to grow malignantly. They found that prevention of neovascularisation led to tumour dormancy. This was seen in epitheliomas transplanted into the irises of rabbits (Gimbrone MAJ *et al* 1972).

Further work concluded that tumour cells have a stimulating influence on endothelial cells, noting that capillaries grow towards tumours from the host unlike skin grafts which send out new capillaries. It was also evident that there were characteristic phases in tumour growth i.e. a pre-vascular phase of minimal growth, followed by a vascular phase of exponential growth. It was at this point that the idea of targeting angiogenesis both directly and indirectly was put forwards as a novel therapeutic goal (Folkman J 1972).

Various techniques were also developed to look at patterns and distributions of tumour vascularity. These ranged from immunohistochemistry to scanning electron microscopy and digital computer technology. Jain and colleagues did extensive studies using some of these techniques which showed that tumour vessels have defective cellular linings, composed of disorganised, branched, overlapping or sprouting endothelial cells. They also showed that openings between these cells contribute to tumour vessel leakiness and may provide a potential route for therapeutic agent molecules (Less J *et al* 1991; Lichtenbeld H *et al* 1996). Other studies looked at the measurement of microvascular density (MVD) in tumours,

thought to have some prognostic significance, supported by the angiogenesis theories to date. The first quantitative evidence was produced by Srivastava et al in 1986 looking at melanoma (Srivastava A *et al* 1986). This showed a positive correlation between the percentage of area occupied by vessels at the tumour-dermal junction and the thickness of the melanoma (r=0.687, p<0.002). However, there was no correlation between vascularity and clinical outcome shown by this study, and these methods have not been adopted as useful prognostic tools. MVD has been studied again more recently as a biomarker for response to anti-angiogenic treatment strategies, with variable results (Willett C *et al* 2005; Jubb A *et al* 2006).

It is now well established that tumour growth and subsequent metastatic spread are critically dependent on the tumours blood supply (Liotta LA and Stracke M 1998; Melero-Martin JM and Dudley AC 2011). Embryonic and tumour tissue are both able to intrinsically build their own blood supplies. In the case of tumours, this is termed tumour neovascularisation, and is made up of angiogenesis, vasculogenesis, intussusception and co-option **(Figure 1.3)**. There may also be an element of chaotic tumour vessel-like formation that allows some blood flow and is otherwise known as 'mimicry' (Sood AK *et al* 2002; Heidemann J *et al* 2006). This is where aggressive tumour cells, rather than endothelial cells, form a pattern of vasculogenic-like networks that are highly permeable and aid in further tumour neovascularisation (Dvorak HF *et al* 1995).

1.2.3   Significance of angiogenesis to tumour growth

Early investigations by Folkman et al into the importance of angiogenesis in tumour growth revealed that for a tumour to grow beyond a threshold size, neovascularization had to precede growth (Folkman J and Klagsbrun M 1987; Folkman J and Shing Y 1992; Folkman J 2003).

Figure 1.3: Mechanisms of tumour vascularisation
A)   Classical angiogenesis – new blood vessels from existing bed
B)   Vasculogenesis by recruitment of CEPs, which differentiate to ECs and establish new microvessels.
C)   Vascular mimicry describes describes small perfused channels within clusters of tumour cells. The term vessel mosaicism refers to microvessels where the continuous endothelium is interspersed with tumour cells.
D)   Vascular co-option is whereby tumour cells surround supporting microvessels (I), causing EC apoptosis. Subsequent hypoxia leads to upregulation of angiogenic mediators by tumour cells, resulting in strong angiogenesis at the periphery of the tumour (III).
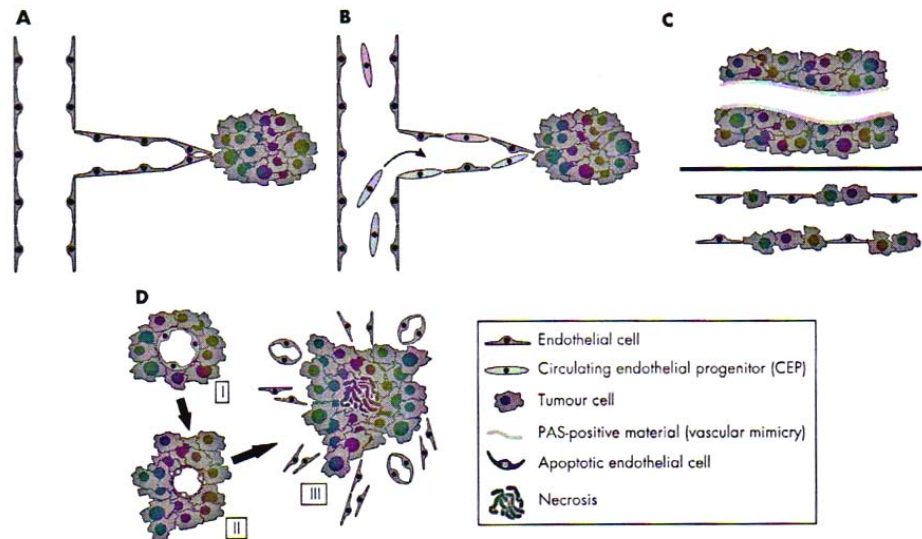
Diagram modified from Heidemann *et al* 2006.

Experiments demonstrated that if angiogenesis is inhibited surrounding a developing tumour, the tumour will not exceed 1 to 2 mm^3 in size; with a diameter which corresponds to the maximum distance oxygen can diffuse. Also, Folkman's theories suggested that without angiogenesis, tumour cells were unlikely to be shed into the circulation and therefore the tumour remained dormant.

Earlier studies on neoplastic transformation focussed on events that occur within transformed cells, but it has since been shown that the pathogenesis of most cancers includes complex and numerous tumour-microenvironmental interactions. The phenotypic switch to angiogenesis, as shown by Folkman and in later studies, is usually accomplished by a subset of tumour cells that induces new capillaries which then converge toward the tumour. These new vessels feed the tumour, and their endothelial cells produce a spectrum of growth factors that have a stimulatory effect on the tumour cells and also a variety of matrix-degrading proteinases that facilitate invasion (Nicosia R *et al* 1986).

But what instigates this change from dormant, avascular tumour, to active, vascular tumour which has a metastatic capacity? Folkman and colleagues had already shown that tumours tend to cluster around capillaries, and that tumours situated far away from a potential blood supply, had a significantly lower mitotic rate (Folkman J 1986; Folkman J 1990). They also showed that the process of a tumour gaining a vascular network relied upon release of pro-angiogenic molecules from the actual tumour cells. These in turn activate certain genes in the surrounding normal host tissue, and hence proteins are produced which are necessary for the production of new tumour vasculature. In this way, neoplastic cells only form a clinically observable tumour if

the host produces a vascular network sufficient to sustain their growth. Furthermore, new blood vessels provide them with a gateway through which to enter the circulation and metastasise (Ribatti D *et al* 2007).

Vascular quiescence in normal tissue is maintained by the dominant influence of endogenous angiogenesis inhibitors over angiogenic stimuli. In health, endothelial cell turnover time is measured in hundreds of days (Ortega N *et al* 1999). There are the occasional exceptions to this e.g. pregnancy and wound healing, where there are transient bursts of blood vessel growth and an increase in endothelial cell turnover to an average of 5 days.

However, in tumour angiogenesis, there is an increased secretion of angiogenic factors and/or downregulation of inhibitors, leading to a tip of the balance **(Figure 1.4).** This concept has since been termed the 'angiogenic switch', whereby a tumour leaves its avascular growth phase, and enters its vascular, exponential growth phase. The balance between these two phases is altered by increasing activator gene expression, changing the bioavailability or activity of the inducer pro-angiogenic proteins, or reducing the concentrations of endogenous inhibitors via changes in gene expression or processing (Bouck N *et al* 1996; Hanahan D and Folkman J 1996). These factors are not only produced from tumour cells, but also from the surrounding stroma and extra-cellular matrix also (Fukumura D *et al* 1998).

When the concept of the angiogenic switch was first introduced, it was termed 'tumour angiogenesis factor' (TAF). It was shown that by blocking its production or targeting its site of action, tumour neovascularisation could be inhibited. In turn, this

Figure 1.4: The Angiogenic Switch

Endogenous factors regulate angiogenesis. The net balance of angiogenic and angiostatic factors controls the extent of angiogenesis. ELR + / - chemokines relates to the presence of a specific amino-acid sequence. ELR+ chemokines induce the migration of neutrophils and are therefore pro-angiogenic.
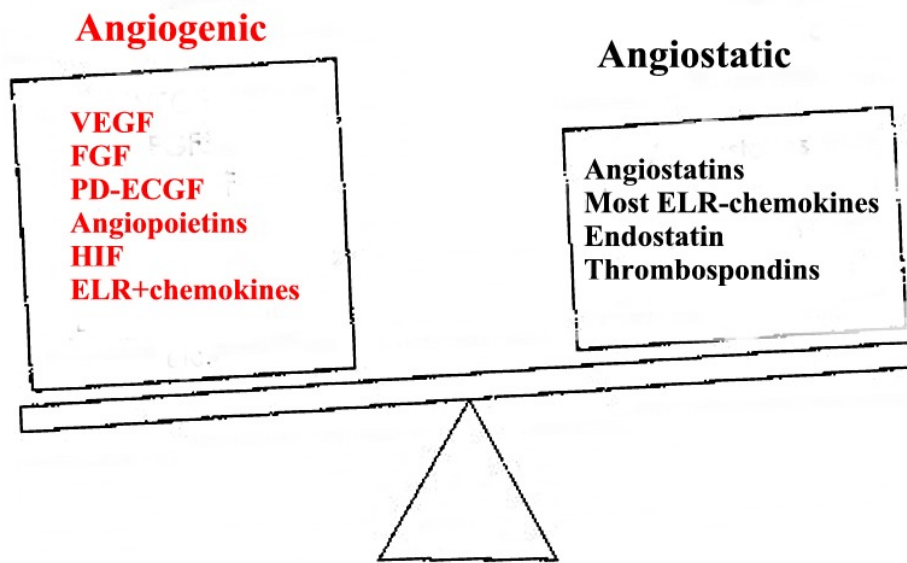
Diagram modified from Heidmann et al 2006.

would prevent expansion or metastatic spread of a tumour, leaving it in a dormant and small state (1-2mm in diameter) (Folkman J 1990). In contrast to these theories, Sakariassen and colleagues showed that it was possible for invasion and angiogenesis to be completely uncoupled in stem-like tumours i.e. tumours with stem-like cancer cells that had no angiogenic potential but still had locomotion (Sakariassen P *et al* 2006).

The predominant stimulus switching on angiogenesis in tumours is thought to be tumour-associated hypoxic conditions. This occurs when tumour mass is critical. Hypoxia leads to activation of hypoxia inducible factor-1α (HIF-1α) which promotes the upregulation of several proangiogenic factors, most importantly vascular endothelial growth factor (VEGF), which will be discussed further in section 1.2 (Rak J *et al* 1995). Other positive regulators of angiogenesis include fibroblast growth factor-2 (FGF-2), IL-8, transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF) and others. These can be exported from tumour cells (Kandel J *et al* 1991), mobilised from the extra-cellular matrix (Vlodavsky I *et al* 1990) or released from host cells (e.g. macrophages) recruited to the tumour (Leibovich SJ *et al* 1987). At the same time, expression of endogenous inhibitors, such as thrombospondin-1 (TSP-1) or interferon beta may be down-regulated (Rastinejad F *et al* 1989; Dameron K *et al* 1994). Thus, the 'angiogenic switch' is a net balance of positive and negative regulators. It is subsequently followed by an angiogenic cascade, which leads to remodelling and formation of vascular channels. This process can be divided into an activation and a resolution phase.

The question then poses itself, as to how and why a tumour develops the capacity to

turn on the angiogenic switch? It has long been accepted that the progression of a tumour reflects their genetic instability and a high mutation rate. Genetic control of the physiological levels of endogenous angiogenesis inhibitors may well be a line of defence against the conversion of dormant tumour cells to a malignant, pro-angiogenic phenotype (Ribatti D *et al* 2007). There is evidence to show that genetic instability must promote the 'angiogenic switch'. Watnick and colleagues observed that the switch in a cell transformation model was dependent on oncogenic RAS expression. They showed that low expression levels of RAS induced cell transformation and increased VEGF (a known pro-angiogenic molecule) expression, along with repression of the anti-angiogenic factor TSP-1 through activation of another key gene, Myc. In this way, oncogene expression was shown to influence the balance and hence the 'angiogenic switch' (Watnick R *et al* 2003).

This concept was also illustrated in another set of experiments carried out by Dameron and colleagues. They established a direct link between the p53 tumour-suppressor gene, tumour angiogenesis and TSP-1. To examine the effect of p53, they cultured fibroblasts from patients with the Li-Fraumeni syndrome who have inherited one wild-type allele and one mutant allele of the p53 gene. When the wild-type allele was lost, these cells acquired potent angiogenic activity coincidental with the loss of TSP-1 production (Dameron K *et al* 1994).

A much debated issue in this field of research is the contribution of bone-marrow-derived endothelial progenitor cells (EPCs) to the process of angiogenesis. Bone marrow-derived stem cells may indeed be a source of EPCs which are then recruited for tumour neovascularisation. In 1997, Asahara and colleagues reported the

isolation of putative EPCs from human peripheral blood, on the basis of cell-surface expression of CD34 and other endothelial cell markers. These cells were reported to differentiate *in vitro* into endothelial cells and seemed to be incorporated into active areas of angiogenesis in various animal models. Ischaemia, along with pro-angiogenic molecules such as VEGF, were reported to contribute to the mobilisation of the EPCs into sites of neovascularisation (Takahashi T *et al* 1999). These cells have subsequently been shown in further studies to 'home-in' and become incorporated into sites of vascular injury and ischaemia (e.g. a tumour microenvironment) (Lin Y *et al* 2000; Rafii S *et al* 2002). More recently, Peters and colleagues analysed the tumour endothelial cells in six individuals who developed cancers after bone-marrow transplantation with donor cells derived from individuals of the opposite sex and found that an average of only 4.9% of cells of the total endothelial cell population were derived from the transplanted bone-marrow (Peters B 2005).

Aside from the bone-marrow source of endothelial cells, the endothelial cell layer of healthy tissue normally remains relatively quiescent, with an estimated turn over period of between 47 and 23,000 days, as shown by labelling studies (Hobson B and Denekamp J 1984). Elevated numbers of circulating endothelial cells have been described in patients with various cancer types, including melanoma, glioma, breast, colonic, gastric and testicular cancer (Mancusco P 2001; Beerepoot L *et al* 2004; Goon *et al* 2006). They appear in the circulation of such cancer patients, either as a result of being shed from localised damaged or activated tumour vessels, or via a more generalised systemic activation - derived from bone-marrow progenitor cells. A number of studies have been carried out to assess the relationship between blood levels of CECs and CEPs and tumour progression in human patients, showing some

interesting results. These will be discussed further in chapter 5. Along with this, the study of angiogenesis has been advanced by the ability to culture endothelial cells *in vitro*, initially using large vessel endothelial cells, such as those isolated from the human umbilical vein (HUVECs), and more recently using micro-vascular endothelial cells (MVECs). These *in vitro* models are discussed further in the following 3 chapters, and realising the limitations of *in vitro* work, can be used in pre-clinical angiogenesis studies, do aid future work.

1.2.4   Background to potential molecular targets and signalling systems

It is clear that angiogenesis is a complex process which relies on numerous factors and steps in order to occur. This creates a vast expanse of potential molecular targets against which agents can be developed in order to inhibit or contribute to the inhibition of tumour angiogenesis. A number of these have been discussed already. This section covers some of the finer details of this area, and how angiogenic pathways can be targeted.

Pro-angiogenic factors are molecules or growth factors such as peptides and lipids, and they fall into two main categories. The first group acts directly on the tumour endothelium to promote mitosis, and the second acts indirectly by signalling cells to release endothelial growth factors. Angiogenic factors are found in all cells, whether involved in new vessel growth or not, and are part of a very intricate regulation system (Larcher F *et al* 1998). As previously mentioned, the tip of the balance of the 'angiogenic switch' towards angiogenesis is the result of genetic instability leading to tumour neovascularisation. This suggests that inhibition of pro-angiogenic molecules

is a potential therapy target.

In the early 1980's the first angiogenic factor was identified, namely basic fibroblast growth factor (bFGF) (Tallquist M *et al* 1999). Since then numerous angiogenic factors have been purified from extracts of tumour cells, cultured endothelial cells and wound fluid, including fibroblast growth factor (FGF-over-expressed in melanoma), vascular endothelial growth factor (VEGF), angiogenin, transforming growth factors (TGF-α and TGF-β), platelet derived growth factor (PDGF) and clotting cascade proteins such as tissue factor (Larcher F *et al* 1998). These act on various protein kinase receptors - for example vascular endothelial growth factor receptor (VEGFR) - which will be detailed in section 1.3. This leads to the stimulation of endothelial cell locomotion and proliferation via an intracellular signalling cascade and resultant activated proteins, all working toward angiogenesis. The protein kinases receptors are therefore also a potential target for anti-angiogenic therapy (Bergers G *et al* 2003).

A protein kinase is a kinase enzyme that modifies other proteins by chemically adding phosphate groups to them (phosphorylation). This usually results in a functional change to the target protein (substrate) by changing enzyme activity, cellular location or association. Kinases are known to regulate the majority of cellular pathways, especially those involved in signal transduction i.e. the transmission of signals within the cell. Because kinases have profound effects on the cell, their activity is highly regulated. If this activity becomes dysregulated, it can frequently cause disease. The kinases in question are the receptor tyrosine-kinases e.g. VEGFR-1, VEGFR-2, FGFR. These receptors are transmembrane proteins, with a tyrosine

kinase domain protruding into the cytoplasm. The extracellular domain serves as the ligand binding part of the molecule, the transmembrane part is a single α helix, and the intracellular domain is responsible for the kinase activity as well as regulatory functions. The active tyrosine kinase goes on to phosphorylate specific target proteins, which are often enzymes themselves. An important target is the Ras protein, a stepping stone to the mitogen activated protein (MAP) kinases signal-transduction chain, leading to down-stream gene-regulatory proteins (Lodish H *et al* 2000). See **figure 1.5** for the VEGF pathway which includes down-stream proteins and shows sites of action of pertinent targeted therapies, including sorafenib – one of the anti-angiogenic agents studied in this thesis.

As previously mentioned, after the discovery of pro-angiogenic molecules and resultant pathways, specific angiogenic inhibitory molecules were also found. This group of endogenous structurally diverse proteins include thrombospondin-1 (TSP-1), interferon α/β, angiostatin, endostatin, antithrombin III, among many others. Many of the precursors of these proteins are components of the extracellular matrix or clotting pathways. Up-regulation of these inhibitory molecules therefore provides another potential target for anti-angiogenic therapy.

VEGF was found to have a central role in angiogenesis. It is a glycoprotein and a potent pro-angiogenic factor which is highly specific for vascular endothelial cells. Endothelial cells express its complementary receptor and it has been identified as a major mediator in angiogenesis (Ferrara N and Henzel 1989). VEGF-A (an isoform) and its receptors are the best characterised signalling pathway in developmental angiogenesis, and loss of a single VEGF-A allele results in embryonic lethality (Yancopoulos G *et al* 2000; Ferrara N *et al* 2003).

Figure 1.5: **The VEGF pathway** - including down-stream proteins and sites of action of targeted therapies, including sorafenib.
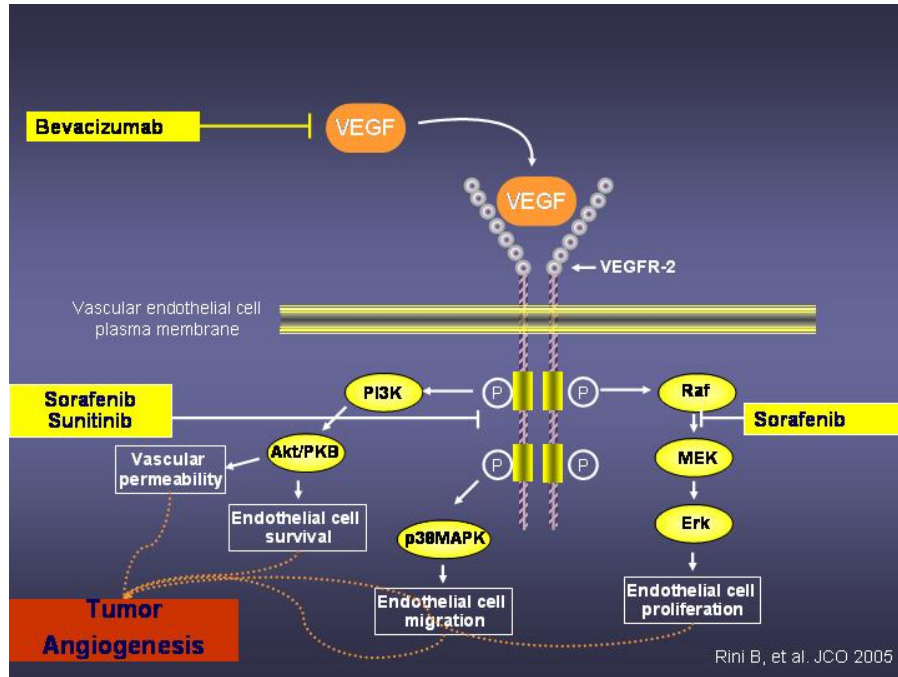
Diagram adapted from Rini B 2005.

Apart from summoning endothelial cells, VEGF acts as an anti-apoptotic and an inductor of vascular fenestrations – hence its former name, vascular permeability factor. In order for it to fulfil its role of new vessel formation, space must be made in the extracellular matrix for the new vessels, which is achieved by matrix metalloproteinases (MMPs). Matrix degradation by MMPs also plays a major part in angiogenesis, and also tumour invasion and metastasis (Hiratsuka S 2002). VEGF isoforms along with MMPs are therefore potential targets for anti-angiogenesis therapy.

There are many different isoforms of VEGF, including VEGF-A as previously mentioned. These correspond to different polypeptide sizes due to alternative splicing of the VEGF messenger RNA (Ferrara N *et al* 2003). Variants mediate their activities through the transmembrane receptor tyrosine kinase family members i.e. VEGFR-1,-2 and -3. VEGFR-2 plays a major role in capillary permeability and angiogenesis, and the other two receptors are important in recruitment of endothelial precursor cells (VEGFR-1), and lymphatic differentiation (VEGFR-3) (Matsumoto T and Claesson-Welsh L 2001; Hattori K *et al* 2002; Karkkainen MJ *et al* 2002). Tumour cells have been shown to over-produce VEGF-A, and inhibition of this can lead to suppression of tumour growth (Ferrara N 2001). Renal cell carcinomas in particular have been shown to express particularly high levels of VEGF-A, providing an explanation for their responsiveness to VEGF-A blockade (Yang J *et al* 2003).

VEGF-A stimulates angiogenesis via VEGFR-1 and -2. The intracellular reactions which allow these receptors to fulfil their roles are both complex and varied. As previously mentioned, the MAP kinase pathway is a prominent signalling pathway, and is induced by VEGF (see **figure 1.5**). Activation of MAP kinase pathway by

VEGF regulates vascular development through the induction of mitogens (chemicals which trigger cell division), inactivation of apoptosis and induction of chemotaxis (Dvorak HF *et al* 1995). It has been found that deletion of genes involved in the regulation or relay of this signalling pathway leads to defective vasculature development during embryogenesis and also tumour angiogenesis (Kranenburg O *et al* 2004). See **Figure 1.6** for a summary diagram showing some of the various strategies to inhibit VEGF signalling. Other isoforms of VEGF, namely VEGF-C and –D, bind and activate their receptor VEGFR-3, and were the first molecules shown to promote lymphangiogenesis. Expression of VEGF-C and –D has been shown to be upregulated in only certain cancers, correlating with increased lymphatic invasion and metastasis (He Y *et al* 2004; Scavelli C *et al* 2004). In a pre-clinical model, mice heterozygous for VEGF-C were shown to develop lymphodema and chylous fluid in the abdomen, suggesting insufficient lymphatic vessels formation. VEGF-C null mice were non-viable (Karkkainen M *et al* 2004).

Another key factor which has been previously mentioned and is co-expressed with the VEGF family in several cancers is hypoxia inducible factor-1 (HIF-1). As tumours grow, some tumour cells, especially central ones within a mass, become hypoxic. The hypoxic signal is crucial to summon a new blood supply. This release of HIF-1 leads to erythropoietin secretion and further angiogenesis stimulation through upregulating several of the proangiogenic factors, including VEGF and basic fibroblast growth factor (bFGF) (Rak J *et al* 1995; Harris A 2002; Semenza G 2002). The degradation of HIF-1 is also dependent on the activation of p53 (a tumour suppressor gene). In some tumours e.g. colorectal and pancreatic, expression of HIF-1 has correlated well

Figure 1.6: Various strategies to inhibit VEGF signalling

Strategies to inhibit VEGF a)monoclonal antibodies targeting VEGF-A

b)+c)monoclonal antibodies targeting VEGFR-1 / 2

d)chimaeric soluble receptors such as the 'VEGF-trap'

e)extracellular inhibitors are aptamers that bind the heparin-binding domain of VEGF165 (pegaptanib).

Other small molecule VEGF RTK inhibitors that inhibit ligand-dependent receptor autophosphorylation of VEGFR-1 and VEGFR-2 are being tested.
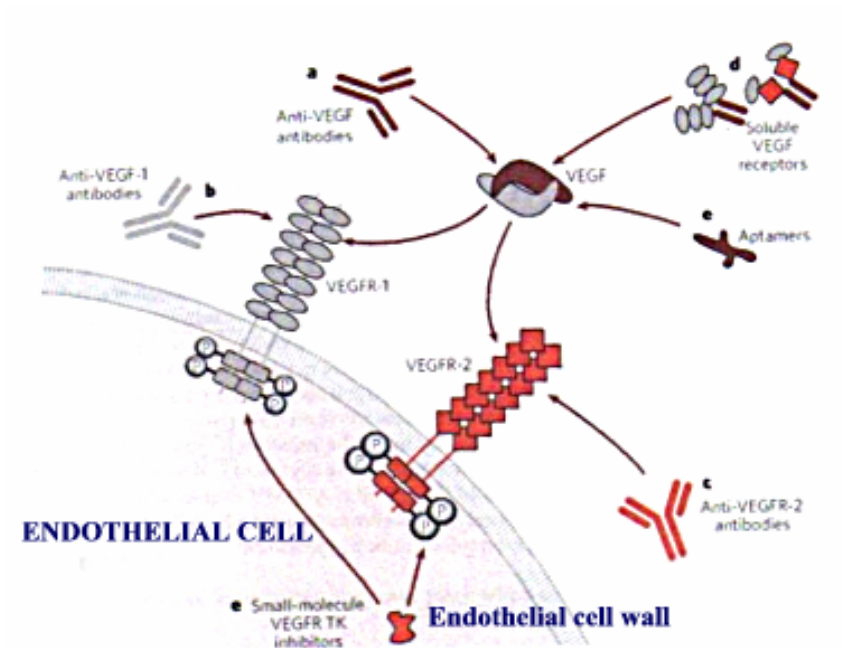
Diagram adapted from Ferrara and Kerbel 2005.

with markers of angiogenesis and prognosis, and degradation of HIF-1 may well be a critical step in the conversion of a dormant tumour to an active one (Safran M and Kaelin W 2003). It is therefore another potentially useful target molecule for drug development (Patiar S and Harris A 2006).

Other signalling molecules that have an established role in the development and differentiation of the vessel wall include platelet-derived growth factor (PDGF-β) and the angiopoietins, which may also be potential therapeutic targets (Yancopoulos G *et al* 2000). PDGF acts on an important receptor group, and functions to regulate smooth muscle cell development. A mutation in one of these receptors e.g. PDFGRβ, leads to inadequate microvasculature, with subsequent haemorrhages and oedema (Tallquist M *et al* 1999; Yu J *et al* 2003). Under normal circumstances these receptors, and their corresponding ligands i.e. PDGF isoforms, promote the development and maintenance of vasculature, and are mainly expressed on pericytes. PDGF-β isoform is required for recruitment of pericytes and maturation of the microvasculature (Lindhal P *et al* 1997). The combination of PDGF and VEGF inhibitors (Sunitinib, Sorafenib, Imatinib) have therefore been proposed as another potentially attractive anti-vascular strategy (Dong J *et al* 2004). These agents have however been linked to increased invasive and metastatic capacity of certain tumours (Ebos *et al* 2009, Paez-Ribes *et al* 2009).

Angiopoietins are ligands (binding molecules) for other important protein kinase receptors namely Tie1 and Tie2, which are similar to VEGFR, and are specific to vascular endothelial cells. Mice lacking Tie1 or Tie2 die due to loss of microvasculature and a reduction in vascular integrity respectively (Dumont D *et al*

1994; Patan S 1998). Both these receptors and their ligands are therefore crucial in endothelial cell assembly, cell-to-cell interaction, endothelial cell survival and extracellular matrix interaction. Inhibition of these angiopoietins and their receptors is therefore another potential anti-angiogenic strategy.

More recently, the role of axon-guidance receptors and ligands has gained much attention in this field. There are four main families of these, but those of particular interest include another group of receptor tyrosine kinases are known as the Eph receptors and their ligands, ephrins (Pasquale E 2005). These have been shown to have an important role in morphogenesis and capillary sprouting. They interact to control cell localisation and enhance vascular development by activating specific molecules (Patan S 2004). The Eph family have also been shown to have major 'locomotion' inducing properties. Interfering with signalling of one of these ligands, namely EphA, has been reported to result in some inhibition of angiogenesis in tumour models (Dobrzanski P *et al* 2004; Astin *et al* 2010). **Figure 1.7** shows some of the above mentioned specific targets in antiangiogenic therapy compared with traditional chemotherapy.

These molecular cascades, some of which have been described above, eventually lead to new capillary formation. This occurs via complex and numerous factor-receptor interactions. Overall, this complex process of angiogenesis requires (as shown in **figure 1.8**):

*1.*Degradation of the basement membrane (of the parent venule) and extracellular matrix around the vessels – *induced by MMPs and other proteases.*

*2.*Endothelial cell migration (chemotaxis) toward the angiogenic stimulus – *induced*

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by VEGF-A, bFGF, angiopoietins, IL-8, VEGF-C, and other proangiogenic molecules acting on their paired receptors.

*3.*Proliferation and alignment of endothelial cells with each other to form a solid sprout *– induced by PDGF isoforms acting on their paired receptors, along with other proangiogenic pathways.*

*4.*Remodelling of basement membrane as vascular tubing reforms and a lumen is seen *– induced by angiopoietins acting at their various tyrosine kinase receptors, along with other proangiogenic pathways.*

Almost every component of the haemostatic system including platelets, endothelial system, coagulation cascade (tissue factor) and fibrinolytic system, also contribute to this process (Auguste P *et al* 2005).

Discovering many of the intricacies of angiogenesis has led to a whole new world of potential therapeutic targets, in both malignancy and various disease processes (Matsuzaki S *et al* 1998; Ribatti D 2010). Multi-targeted, patient-friendly regimens, aimed at the molecular make-up of a particular tumour and its vasculature are being introduced into the clinical setting, and hold promise of a shift away from conventional cytotoxic regimens. This is a major concept shift in cancer treatment as we move closer toward understanding tumours at a molecular level and understanding processes that may produce 'controlled disease states' with the patient surviving with their cancer for ever lengthening periods of time.

Figure 1.7: Targets in antiangiogenic therapy versus traditional chemotherapy

The multitude of potential antiangiogenic specific cellular targets allows for more diverse mechanisms of action.

Chemotherapy targets   Antiangiogenic targets

Growth factors
DNA
DNA
Receptors/tyrosine kinase
Adhesion molecules
Topoisomerases   TUMOUR   Enzymes
Proteases
Integrins
Microtubules
Microtubules
Membrane glycoproteins
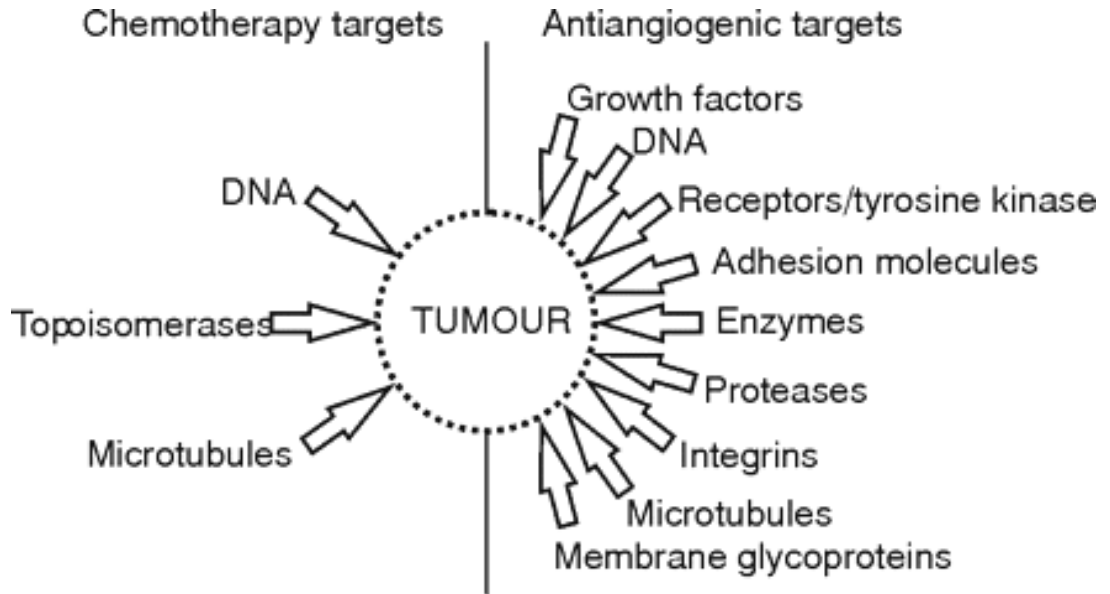
Diagram modified from Kerbel 2001.

Figure 1.8: Summary of stages in angiogenesis.

Diagram to show degradation of the basement membrane and extracellular matrix around the vessels, followed by endothelial cell migration toward the angiogenic stimulus. Proliferation and alignment of endothelial cells then occurs to form a solid sprout. This is followed by remodelling of the basement membrane as vascular tubing reforms and a lumen is seen.

1.2.5　Inhibition of angiogenesis as anti-cancer therapy

The role of anti-angiogenic therapy in cancer treatment is based on a number of theories:-

▪ It is less toxic and more specific in its target

▪ There is no need for regular breaks in treatment to allow healthy tissues to recover as it is specifically targeted to tumour endothelium with minimal adverse effects elsewhere in the body compared to convetional chemotherapy.

▪ It can be used to reduce the repopulation of cancer cells during the intervals between traditional chemotherapy / radiotherapy.

▪ It may avoid cumulative toxicities of traditional cytotoxic regimens and enables the possibility of adding agents into a regimen, hence avoiding overlapping toxicities.

Inducing apoptosis in endothelial cells with stable machinery i.e. a stable cellular configuration, is easier than inducing apoptosis in cancer cells with unstable machinery. This is also a way of avoiding the development of resistance.

Many angiogenesis inhibitors are currently in clinical trials and over ten have now been FDA approved (Ribatti D 2010). Alongside the development of such agents, there is also a new class of agents known as 'vascular-disrupting' drugs e.g. that can cause acute vascular occlusion and disruption of tumour blood flow (Siemann D *et al* 2004). Numerous specific anti-angiogenic molecules have been created by biotechnology and pharmaceutical companies. **Table 1.1** summaries their phase of clinical development and principal molecular targets.

Table 1.1: Summary of principal molecular targets of novel anti-cancer and anti-angiogenic agents – their phase of clinical development, with indications approved (if any) (adapted from Tourneau C at al 2008)

Drug	Company	Phase of clinical develop -ment	Indications approved	Principal molecular targets
Imatinib	Novartis	III	ALL,CML, GIST	PDGFR, KIT, BCR-ACL
Avastin	Genentech	III	mCRC, NSCLC, breast	VEGF
Sunitinib	Pfizer	III	GIST,RCC	VEGFR, PDGFR, KIT, FLT3, RET
Sorafenib	Bayer	III	RCC	VEGFR, PDGFR, KIT,RET, RAF
Dasatinib	Bristol-Myers Squibb	III-CML II -solid	ALL,CML	PDGFR, KIT
Vatalanib	Novartis/Bayer	III		VEGFR, PDGFR, KIT
ZD6474	AstraZeneca	II		VEGFR, EGFR, RET
Axitinib	Pfizer	II		VEGFR, PDGFR, KIT
GW786034	GlaxoSmithKline	II		VEGFR, PDGFR, KIT
AZD2171	AstraZeneca	II		VEGFR, PDGFR, KIT
AEE788	Novartis	I		VEGFR, EGFR
BIBF1120	Boehringer	I		VEGFR, PDGFR, FGFR, SRC
BMS582664	Bristol-Myers	I		VEGFR, FGFR
AMG706	Amger	I		VEGFR, PDGFR, KIT, RET
CHIR258	Novartis	I		VEGFR, PDGFR, KIT,FGFR, FLT3
BAY 57-9352	Bayer	I		VEGFR, PDGFR, KIT
XL999	Exelixis	I		VEGFR, PDGFR, KIT,FGFR, FLT3, SRC
XL820	Exelixis	I		VEGFR, PDGFR, KIT

Key: ALL – acute lymphoblastic leukaemia; CML – chronic myeloid leukaemia; GIST – gastrointestinal stromal tumour; RCC – renal cell carcinoma. * AEE788 also inhibits HER2.

These molecules are produced by:

▪ The identification of a target molecule and development of an antibody to (e.g. anti-VEGF antibody) or a synthetic inhibitor of receptors (e.g anti-VEGF-A monoclonal antibody – bevacizumab, and tyrosine kinase inhibitor – sorafenib (Ferrara N *et al* 2004; Wilhelm S *et al* 2004).

▪ The discovery of anti-angiogenic properties of an already existing drug (like thalidomide and paclitaxel (Browder T *et al* 1995) – discussed further in section 1.3.

▪ The discovery of a specific endogenous anti-angiogenic molecule (like endostatin-fragment of collagen XVIII - a specific endothelial inhibitor) (Heljasvaara R *et al* 2005).

Figure 1.7, as previously mentioned, shows the diversity of targets in conventional chemotherapy and antiangiogenic therapy of human tumours. The multitude of potential anti-angiogenic cellular targets permits more diverse mechanisms of action compared with conventional chemotherapy (Kerbel R and Folkman J 2002).

The unique structural features of tumour vasculature mean that, in general, tumour angiogenesis can and should be targeted, and promising results of clinical trials have supported this. FDA approval or NCCN recommendations have already been made for some specific antiangiogenics to be used in conjunction with existing regimens as first line treatments, e.g. bevacizumab in conjunction with IFN in renal cell carcinoma (Rini B *et al* 2004; Escudier B *et al* 2008) or as single agents (Yang *et al* 2003). Interestingly, the results of angiogenesis research have also led to the use of anti-angiogenics in diseases characterised by over-exuberant angiogenesis such as neovascular and exudative ocular diseases. The results of clinical trials have now led

to FDA approval and the incorporation of ranibizumab-derived from bevacizumab-into the NICE guidelines for treatment of wet age-related macular degeneration (Rosenfold P *et al* 2006) .Successes in treating such diseases are helping to push forward the boundaries in the area of cancer, which is so crucially needed. **Table 1.1** gives a summary of the principal molecular targets of the targeted anticancer agents, with their approved indication (Tourneau C *et al* 2008).

Despite the initial presumption that endothelial cells would remain relatively stable to such treatments, acquired resistance has been associated with anti-angiogenic therapies. There has been emerging evidence that, in the case of VEGF-A inhibition, other angiogenic pathways will eventually override as the disease progresses. Other possible mechanisms for acquired resistance include selection and overgrowth of tumour cell variants that are 'hypoxic resistant' and are thus less angiogenesis-dependent (Kerbel R 2001; Sweeney C *et al* 2003). Other studies have suggested that, in some cases, endothelial cells associated with tumours are not genetically stable, and have demonstrated cytogenetical abnormalities (Hida K 2004; Bergers G and Hanahan D 2008).

Consideration of all these factors needs to be taken into account in devising successful and multi-targeted cancer treatment strategies for the future.

1.3 Metronomic Chemotherapy

1.3.1   Basic concepts

Metronomic chemotherapy is one area of active research into novel cancer treatment concepts. It has received much attention in the medical literature over recent years. As efforts continue to develop more targeted and user-friendly cancer regimens, there

is increasing pre-clinical evidence to support the notion that 'metronomic' type of dose scheduling of conventional chemotherapy agents may become a clinically important modification.

Metronomic is derived from the Greek words 'metron' – to measure and 'nomos' – to rule or divide. The English translation is 'of or relating to a metronome', also meaning unvaryingly regular in rhythm. The term metronomic in this context is given to continuous i.e. daily low-dose regimens of chemotherapy. It has similarly been associated with rhythmic musicians and their music, and ticking clocks. It is therefore seemingly obvious why the term metronomic was originally given to this type of treatment regimen.

However, 'metronomic' simply explains the way in which these regimens are administered. It does not reflect the molecular or clinical justifications for these regimens, and may indeed be a confusing misnomer. This section highlights the potentially confusing terminology, discusses the justification for specific and targeted chemotherapy regimens, and summarises the new advances in 'metronomics'.

Conventional chemotherapy was intended to kill or inhibit as many cancer cells as possible. As previously explained, the design of new drugs had traditionally been focussed on the destruction of cancer cell DNA and utilising cancer cell proliferation to take effect (Skipper HE *et al* 1970). The maximum-tolerated dose approach therefore came in to widespread use and the design of most chemotherapy regimens has relied on this model ever since.

Around the same period, as previously mentioned in section 1.2.2, Folkman described

a new target for cancer therapy by introducing the concept of angiogenesis. The evidence suggested that tumour growth was dependent on the development of a blood supply by the process of angiogenesis (Folkman J 1971). The vascular endothelial cells, their surrounding stroma and associated factors, were therefore recognised as a potential target for anti-tumour therapies.

In 2000, Eberhard et al and Klement et al independently showed the presence of dividing endothelial cells in newly forming tumour blood vessels. It was anticipated that these endothelial cells – being genetically stable - would remain sensitive to chemotherapy, regardless of tumour cell resistance (Eberhard *et al* 2000; Klement G *et al* 2000). This added further weight to the development of anti-angiogenic therapy. Postulating this type of anti-cancer mechanism also gave theoretical justification to the concept that drug doses, well below the original cytotoxic ranges of bolus MTD approaches, could either become or be incorporated into anti-cancer regimens. This change in actual chemotherapy administration was found to reduce adverse effects significantly, with resultant improved benefit to toxicity profiles in preclinical models and favourable effects on patients' quality of life in early clinical studies (Browder T *et al* 2000; Shaked Y 2005).

The term 'metronomic' in relation to chemotherapy, was first described by Hanahan and colleagues in 1996 (Hanahan D and Folkman J 1996). This study looked at the effects of using continuous low-dose (10-33% of maximum tolerated dose –MTD) cytotoxic agents, without any extended rest periods. It showed that total dose achieved could be higher than MTD by using this style of drug administration. Gasparini showed similar results, again using 10-33% of MTD (Gasparini *et al* 2001).

Work carried out by Browder et al in 2000, showed that continuous low-dose cytotoxic agents could inhibit tumour angiogenesis (Browder T *et al* 2000). This was seen in mice bearing different solid tumours. It was also around this time that the term metronomic became synonymous with anti-angiogenic, or anti-endothelial. In the same year as Browder's work, Klement showed that cyclophosphamide and vinblastine could be exploited for their antiangiogenic effects when given as continuous low-dose regimens in vivo, with promising results (Klement G *et al* 2000). Kerbel then went on to show that a variety of single chemotherapeutic agents when given in low doses, had the common ability of inhibiting angiogenesis, but also inhibited tumour growth (Kerbel R 2000). Since then, a number of *in vitro* studies (see **table 1.2**) have shown more promising data on the selective responsiveness of endothelial cells towards metronomic schedules (Bocci G 2002; Grant DS 2003). This added further weight to the move towards metronomics, although proof of efficacy in the clinical setting was yet to be seen. See **Table 1.2** for a summary of *in vitro* studies showing selective responsiveness of endothelial cells to metronomic chemotherapy.

Studies listed in **Table 1.2** reported no evidence of cytotoxicity on endothelial cells, although their proliferation was inhibited. Bocci et al did however report apoptosis in cells treated for over 6 days, which may be explained by an indirect mechanism of an imbalance between inhibiting and proangiogenic molecules (Bocci G *et al* 2002; Kerbel RS and Kamen 2004). One of the potential mediators of the anti-angiogenic properties of metronomic chemotherapy is thrombospondin 1 (TSP1) – a well known endogenous angiogenic inhibitor (Lawler J 2002). TSP1 was shown to increase dramatically following continuous dosing with cyclophoshamide in *in vitro* vascular endothelial

Table 1.2: Summary of in vitro studies of endothelial cells -responsiveness to
metronomically dosed chemotherapy.

*Key:*IC$_{50}$=concentration of drug required to inhibit 50% of cell growth

Drug regime & Reference	Assay	Reported response
1.Methotrexate (Hirata S 1989)	Inhibition of proliferation	EC inhibited by low concs (5nM)
2. Paclitaxel (Belotti D 1996)	Inhibition of proliferation, motility and cord formation	Inhibition of EC proliferation (IC$_{50}$=0.5-4nM) and chemotaxis & invasiveness (10pM)
3. Vinblastine (Vacca A 1999)	Inhibition of proliferation,migration and MMP secretion	ECs inhibited by ultra-low concs (0.1-1pM), leucs, fibroblasts and tumour cells not inhibited
4. Paclitaxel & Vinblastine (separately) (Klement G 2002)	Inhibition of proliferation	HUVEC proliferation inhibited (IC$_{50}$=0.4-0.5nM for both drugs); tumour cells inhibited by IC$_{50}$=2-27nM in monolayer culture & 3.4-10.1µM in spheroid culture
5. Paclitaxel, Cyclophosphamide & Epothilone B (separately) (Bocci G 2002)	Inhibition of proliferation and induction of apoptosis	Daily exposure of individual drug over 6 days inhibited EC proliferation (IC$_{50}$=50-100pM); IC$_{50}$ values for tumour cells and fibroblasts at least 10x more than for EC; induction of apoptosis only detected in EC
6. Paclitaxel (Wang J 2003)	Inhibition of proliferation and tube formation	EC selectively inhibited over 3 days at extremely low drug concentrations (0.1-100pM; IC$_{50}$=0.1pM); 6 different non-EC types inhibited at

		10^4-10^5 fold higher concentrations (IC_{50}=1-10nM); EC tube formation also inhibited in vitro.
7. Paclitaxel & Docetaxel (separately) (Grant DS 2003)	Inhibition of proliferation, migration and capillary sprouting	EC found to be 10-100x more sensitive than tumour cells; docetaxel 10x more effective than paclitaxel.
8. Temozolomide (Kurzen H 2003)	Inhibition of proliferation, adhesion and tube formation	HUVEC proliferation on Matrigel inhibited (5mM), proliferation and adhesion inhibited x5 higher concs.
9. Adriamycin, Idarubicin, FU, Paclitaxel, Etoposide (separately) (Drevs J 2004)	Inhibition of proliferation	HUVEC proliferation inhibited at 4.02M
10. Temozolomide, Estramustine, Paclitaxel, Compound 5h (Lam T 2007)	Inhibition of proliferation and apoptosis	Estramustine and temozolomide inhibited HUVECs with IC_{50} of 4.5µM and 6.6µM respectively, enhanced by antiangiogenic

cells (Hamano *et al* Y 2004). Hamano's group also compared TSP1 deficient mice with normal mice, both bearing melanoma. They found that a metronomic schedule of cyclophosphamide caused melanoma regression in the normal, but not in the TSP1 deficient mice. Similar results were seen by Bocci and colleagues when comparing TSP1 deficient to normal mice, both bearing Lewis lung carcinomas and treated with metronomic dosing of cyclophosphamide (Bocci G *et al* 2003).

1.3.2 Therapeutic implications

The current thinking is that by utilising the anti-angiogenic action of chemotherapeutics in combination with specific molecularly targeted drugs, tumours can become a chronic, stable disease process, whereby tumour load is kept to the lowest possible level (Hahnfeldt P *et al* 2003). Traditional regimens have very rarely led to tumour regression or eradication in solid adult malignancies. This is why the anti-angiogenic approach leading to, if not remission, at least stable disease is looking more and more appealing. It has long been known that survival need not be incompatible with the presence of tumour, as long as its growth is kept under control. This was shown in work by Takahashi emphasising that cytostatic as opposed to cytotoxic regimens made the most impact on patient survival (Takahashi Y and Nishioka K 1995; Takahashi Y *et al* 2000).

However, the traditional method of giving conventional cytotoxic agents in several intense cycles remains the mainstay of treatment for adult solid malignancies. There are legitimate concerns that the methods we currently use to achieve the conventional cytotoxic effects of chemotherapy more often than not have detrimental effects on the patient and also on the tumour. The long rest periods given between these traditional

cycles to allow recovery of susceptible organs e.g. gastrointestinal tract and bone marrow, have since been found to actually encourage tumour angiogenesis and therefore tumour growth (Hannahan D *et al* 2000). Not only this, but such non-specific cytotoxic regimens often lead to severe adverse effects and greatly impair quality of life. There is also evidence that the prothrombotic status that chemotherapy agents induce results in an acute phase reactant profile that may not only have pro-thrombotic but also pro-angiogenic effects (Hembrough T *et al* 2003).

Since the publication of the results of a clinical trial of metronomic cyclophosphamide and methotrexate for the treatment of advanced breast cancer by Colleoni et al 10 years ago (Colleoni M *et al* 2001), numerous studies evaluating variations of this approach in breast cancer (Bottini A *et al* 2006; Colleoni M *et al* 2006), prostate (Glode L *et al* 2003) and ovarian cancer (Garcia A *et al* 2008) among other tumour types, have been reported (Kerbel RS and Kamen 2004; Gille J *et al* 2005). All these studies confirm the excellent safety profile of metronomic chemotherapy, and results have generally warranted further clinical evaluation. With the proviso that these results are not all from phase III randomised trials yet, there are nevertheless promising tumour suppressive effects that have been reported, particularly in renal cell carcinoma, breast and lung cancer (Klement G *et al* 2000; Takahashi Y *et al* 2000; Kurzen H *et al* 2003; Burstein H *et al* 2005).

Randomised phase III trials however are necessary for this concept to gain widespread acceptance. There is however, a potential limitation in the developmental design of these early clinical trials. They have been carried out with a stated metronomic intent, but no obvious predating developmental work was done to define

the dosing schedule used to determine the clinical metronomic dose. As previously stated, most of the commonly used anti-cancer chemotherapeutic agents have now been shown to be capable of inhibiting angiogenesis (mainly *in vitro*) when given in 'metronomic' dosing schedules (Miller KD *et al* 2001; Kerbel R and Folkman J 2002). Research ambiguity exists in this field since it is first necessary to define what dose or what dose-range is actually 'metronomic', be it in the clinical or pre-clinical setting. Many of the supposedly 'metronomic' trials have simply used an empirically derived extended dosing schedule with predominantly cytotoxic effects. Miller laid down some criteria in that any chemotherapeutic agent which required a higher dose to inhibit or kill endothelial cells than that required for tumour cells would not be classed as anti-angiogenic (Miller KD *et al* 2001). This however, is also ambiguous as many agents do not have extensive phase I data to define a distinguishable 'cytotoxic' extended dosing schedule and therefore give the opportunity to distinguish this from a metronomic schedule.

A different approach to these areas of ambiguity, at least in the clinical sense, has been proposed by Maraveyas et al (Maraveyas A *et al* 2005). The theory proposed, based on *in vitro* endothelial cell studies, is that there is a dose-response effect on the endothelium, even in the sub-clinical dose range. It is therefore not always appropriate to use an arbitrary 10-33% of the conventional dose. Also, given that bone marrow suppression is a proangiogenic stimulus, the clinical metronomic dose should be the maximum dose that can be delivered in a metronomic schedule without detectable bone marrow perturbation or induction of other proangiogenic stimuli such as activation of the coagulation pathway. This would potentially allow for a more principled approach to the delivery of metronomic chemotherapy, and is the

underlying theory behind both the *in vitro* and clinical studies of this thesis. The clinical study written up in this thesis shows, for the first time ever, the splitting of a conventional level schedule to a metronomic schedule based on dose-response (Maraveyas A *et al* 2005; Lam T *et al* 2006)

1.3.3 Rationale behind combining metronomically-dosed traditional chemotherapy with specific angiogenesis inhibitors

It has become increasingly likely that cancer therapy, with a few exceptions, will need to be combinatorial. It seems logical to target multiple pathways simultaneously. Combination regimens have been traditionally based on the avoidance of toxicities common to different agents and also on modest cancer-specific affects seen in the early stages of a drug's development. While the number of agents available in the early days of cancer treatment was slow to increase, this empirical approach remained unchallenged.

However, the last decade has seen the emergence of multiple novel agents with potential anti-cancer effects. Only a small proportion of these have been developed as chemotherapy in the conventional sense, while the majority have been developed as oral agents that are molecularly-targeted. With the rate of novel agent development, the number of potential double and even triple combinations will become so large that not all the mice in the world, let alone humans would allow for potential study of possible regimens.

One of the most prolific areas within agent development and testing has been that of angiogenesis inhibitors as previously described in section 1.2.5 (Ferrara N and Kerbel

2005). Furthermore, many conventional 'older' agents are now having anti-angiogenic properties attributed to them e.g. taxanes (Belotti D *et al* 1996) using the metronomic approach. Combinational anti-angiogenic protocols have been used *in vitro* and *in vivo* studies, and have been shown to be more effective than single agent regimens (Retter A *et al* 2003; Longoria R *et al* 2005). The mechanisms of these synergistic or addititive anti-tumour effects seen in such combination regimens are not clear, in that it would seem counterintuitive that 'tumour-starving' anti-angiogenic drugs that suppress blood flow in tumours actually increase the efficacy of chemotherapy (Ferrara N and Kerbel 2005).

However, the realisation that single agent anti-angiogenic treatments are unlikely to have the sustained therapeutic impact wished for anti-cancer treatment in many solid malignancies has meant that combinations regimens are now considered to be the way forward (Retter A *et al* 2003).What is not clear is whether in this setting, combinations have necessarily got to include chemotherapy conventionally dosed or in a low-dose metronomic fashion. Metronomic dosing is suitable for combinations of traditional cytotoxic drugs, especially those that are now being made available in oral form e.g. paclitaxel, as they can be administered easily with these newer molecularly-targeted anti-angiogenic agents, the majority of which are available as orally administered agents. The paradigm from HIV and the multiplicity of the oral agents taken daily is obvious. Succesful combinations could become composite formulations in the future. Both pre-clinical and clinical evidence suggests that the anti-angiogenic effect of metronomic chemotherapy can be enhanced by the administration of various specific and molecularly-targeted agents (Kamen B *et al* 2000; Burstein H *et al* 2005; Longoria R *et al* 2005; Buckstein R *et al* 2006; Orlando L *et al* 2006; Lam T *et al*

2007). It is through these studies that the field of multi-targeted cancer therapy continues to grow.

As ±previously mentioned, the vast proliferation of available anti-angiogenic agents, leads us to conclude that the combinatorial permutations far outstrip our capacity to study these through conventional clinical trial (Dark G *et al* 1997; Clark J *et al* 2005; Ribatti D 2010). There is therefore a growing need for the development of predictive tools to allow selection of the most promising combinations suitable for further clinical development. It is also essential to identify suitable cytotoxic agents for the metronomic approach and for each agent, to establish the optimal dose at which angiogenesis inhibition occurs (Parulekar WR and Eisenhauer E 2004; Maraveyas A *et al* 2005). Along with this, to determine drug resistance status in metronomic regimens and develop surrogate markers used to measure treatment success e.g. circulating endothelial cells as discussed in chapter 5 (Gasparini *et al* 2001; Bhatt R *et al* 2007).

Many studies to date of the metronomic approach have been based on animal models, and these studies claim to allow for the detection of pharmacokinetic and pharmacodynamic properties, making one combination more promising than another. There are also advantages of animal models when it comes to studying potential effects of sequential rather than concurrent administration of agents. However, with the vast number of currently available agents - many for whom mechanism of action is poorly understood - there remains a problematic dimension in initiating animal studies when *in vitro* work clearly demonstrates no potential synergy in combinatorial studies. Infact, in some cases antagonistic effects of potential combinations have been

shown on target cells (Budman DR *et al* 2002; Lam T *et al* 2007). It is for these reasons that the use of endothelial cell models can provide a useful stepping stone to the clinic, and provide relevant data on potentially synergistic combinations in this potentially confusing era of multi-targeted therapy.

1.3.4   Proof of concept of metronomic dosing and combination regimens

Proof of concept of any type of therapy schedule, in this case 'metronomic dosing', requires the development of appropriate intermediate surrogate markers, which can be monitored before and after therapy. Since the avoidance of bone marrow perturbation is essential in the delivery of metronomic chemotherapy, bone marrow stem cells can be measured to show response to treatment. Development of cheap and reproducible assays for these potential surrogate markers, along with others are therefore crucial if this treatment strategy is to be successful (Bertolini F *et al* 2006).

 Another important consideration is that the concept of longest possible drug exposure requires careful assessment of the drugs involved, their pharmacokinetics (Kamen B *et al* 2000) and the cumulative or separate effects of metabolites. The aim is to minimise resistance and prevent regrowth of tumour cells between administrations (Norton L 1997; Kim JJ and Tannock I 2005; Shaked Y *et al* 2006). Early clinical results are varied, depending on the drug used and the tumour type. Drugs which have been relatively extensively studied in the metronomic approach to date include alkylating agents e.g. cyclophosphamide, taxanes, anthracyclines and vinca alkaloids (Johnson DH and Carbone D 1997; Seidman AD *et al* 1998; Miller K *et al* 2001; Colleoni *et al* 2002). However, the ability of metronomic chemotherapy to block angiogenesis in terms of effective prolongation of patient survival remains to be

proven in randomised phase III trial settings.

Pre-clinical scientific evidence is necessary to provide rationale behind the selection of combination regimens. For example, synergism when angiogenesis inhibitors are combined with cytotoxic agents given in a metronomic regimen has been seen in early pre-clinical work (Gasparini G *et al* 1994; Kakeji Y and Teicher B 1997). The synergistic effects in these studies were thought to be via the parenchymal and stromal targets of the cancer. Results of further studies by Browder and Klement show that the combination of selective angiogenesis inhibitors with metronomic scheduling of chemotherapy can improve the effects of these agents on intratumoral vasculature, enhancing therapeutic benefit. Klement and colleagues combined continuous low dose vinblastine with a monoclonal antibody that neutralised VEGF-2 receptor (Klement G *et al* 2000; Klement G *et al* 2002). Similarly, Lam et al showed synergistic activity between estramustine and temozolomide when combined with anti-VEGFR-2 antibody, leading to inhibition of human umbilical venous endothelial cells (Lam T *et al* 2007). This pre-clinical work is crucial to the logical and rational development of combination regimens.

The aim of this relatively new therapeutic strategy, whereby metronomic schedules of chemotherapy are combined with molecularly-targeted compounds, is to achieve a long-term dormant state in residual tumour focus, and minimise treatment side-effects (O'Reilly MS *et al* 1996). In the end, clinical trials are going to have to demonstrate a significant improvement in hard endpoints, such as overall survival or progression free survival. Simply reducing toxicity and making the patient feel better, but also abrogating efficacy, is not an option.

The major challenge is to continue to develop suitable biological markers to monitor treatment responses, and to have a standard approach to testing suitable combination regimens in the pre-clinical setting that will potentially induce a beneficial effect in the clinical setting. This therapeutic effect should be aimed at disease control rather than cure.

1.4   Melanoma

1.4.1   Definition

Malignant Melanoma is a malignant tumour arising from uncontrolled growth of melanocytes. These are pigment-producing cells, predominantly found in the skin, but also in the bowel and the eye. Melanocytes are neural crest derivatives and in the skin, these usually reside in the stratum germinativum, which is the lower layer of the epidermis. It is this layer which contains actively dividing cells. Melanocytes have a characteristic colour due to their production of the dark pigment 'melanin'. Melanin acts to protect the surrounding skin by absorbing UV light.

Malignant melanoma occurs when melanocytes undergo transformation into the malignant phenotype either within a preexisting mole (naevus) or de novo. In the clinical situation, change in size, shape or colour may make one suspicious of malignant change within a mole. Also, minor changes in diameter, signs of inflammation, itching, crusting or bleeding all make one suspicious.

1.4.2   Background

Malignant melanoma makes up 3% of all cancers worldwide. Whilst it accounts for only 5% of all skin cancers, it causes the majority (75%) of skin cancer related deaths (Boring C 1994, CancerResearchUK 2011). Despite decades of research, the greatest

chance of cure is in early resection of thin tumours. According to the WHO report, there are about 48,000 melanoma related deaths worldwide per year (Lucas R 2006) and the overall incidence is currently estimated at 1 in 75. This has risen drastically over the last few decades, and continues to rise annually (Thompson JF *et al* 2005). Caucasians living in Australia have a 1 in 14 incidence, compared to a 1 in 80 incidence in the UK. Survival due to melanoma has been improving mainly due to early detection and currently ranges from 67% for black men to 93% for white women in the US (Thompson JF *et al* 2005).

Risk factors for developing a Malignant Melanoma include:

▪ Premalignant lesions (in 7% of the population)

▪ Previous melanoma

▪ Age (increasing age associated with increasing incidence)

▪ Race (rare in Afrocarribean and Asian population)

▪ Economic status (higher economic status increase risk)

▪ Fitzpatrick Type I skin (Caucasian, blond or red hair, freckles, fair skin, blue eyes, very sensitive to sun – always burns and never tans)

▪ Sunburn (four times more likely to get melanoma if episodes of sunburn under 10 years of age)

There are different histological types of melanoma which, among others, include superficial spreading (around 70% of all melanomas), nodular, melanoma in lentigo, acral lentiginous, amelanotic, desmoplastic. Each type has a different presentation and varying degrees of aggression.

Median survival of patients with distant metastases is 30 weeks, and the majority of patients with such advanced disease will be dead at 2 years as per the NCCN. Despite continued research efforts into devising effective chemotherapeutic schedules for

patients with advanced disease, results have remained disappointing. Complete durable responses to systemic therapy occur in only a small minority. The development of better treatment regimens is therefore of utmost importance. The aim is to achieve higher response rates, extended time to progression of disease with reduced toxicity and greater ease of administration.

1.4.3 Diagnosis and Staging

Diagnosis of melanoma is made up of clinical evaluation of the patient and a histological examination of the lesion in question. Diagnosis should be made as promptly as possible to ensure early and effective treatment. Clinical features suggestive of malignant change within an existing or new skin lesion include change in size, shape or colour, and also itching, bleeding and inflammation. A popular method has been advocated to assist in recognising signs and symptoms of melanoma in the form of a mnemonic: 'ABCDE' (Abbasi N *et al* 2004)

▪ Asymmetrical skin lesion

▪ Border of the lesion is irregular

▪ Colour: usually multiple colours rather than uniformly pigmented

▪ Diameter: greater than 6mm is more likely to be melanoma than smaller lesions

▪ Enlarging: enlarging or evolving

A dermatoscope is used by the clinician in the clinic to aid in the diagnosis and it is important to remember that malignant melanomas often defy the 'ABCDE' mnemonic completely. The presence of any of these features does however increase the likelihood of a lesion being a melanoma and **figure 1.9** shows examples of this mnemonic illustrated. Diagnosis requires experience, as early stages may resemble harmless moles or be completely without pigment. If there is any suspicion, then the

Figure 1.9: ABCDE in aiding melanoma diagnosis (adapted from Abbasi 2004)

 A stands for ASYMMETRY; one half unlike the other half.

 B stands for BORDER; irregular, scalloped or poorly defined.
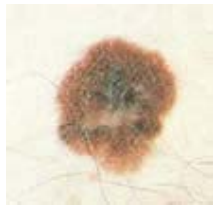
 C stands for COLOUR; varied from one area to another; shades of tan, brown and black; sometimes white, red or blue.
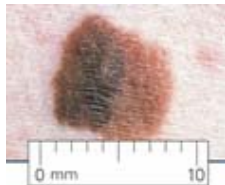
 D stands for DIAMETER; melanomas are usually greater than 6mm (size of a pencil eraser) when diagnosed, but they can be smaller.

E stands for EVOLVING; a mole or skin lesion that looks different from the rest or is changing in size, shape or colour.

primary management is to perform an excision biopsy. This provides the clinician with a histological diagnosis aswell as providing initial treatment for the tumour. The preferred surgical margin for the initial excision biopsy should be narrow (1-2mm) to prevent disruption of lymphatic drainage (Swanson N *et al* 2002). If the histological diagnosis of melanoma is comfirmed, the pathologist will provide a detailed information on the depth of tumour invasion using 'Breslow thickness' and 'Clark's level', which are both indicative of tumour depth and linked to prognosis. Depth of invasion was first reported as a prognostic factor in 1970 by a surgeon named Dr.A.Breslow (Breslow A 1970)   Breslow thickness is the distance between the granular layer of epidermis and deepest part of the melanoma **(Figure 1.10)** using an ocular micrometer at a right angle to the skin. It is directly related to survival and is included in the AJCC staging guidelines as a major prognostic factor (Balch CM *et al* 2001; Balch CM *et al* 2009). Clarks level describes the level to which tumour has infiltrated – level I (just epidermis), level II (papillary dermis), level III (junction between papillary and reticular dermis), level IV (into reticular dermis), level V (into subcutaneous fat). The feature of ulceration of the primary lesion has also been added to pathological staging criteria for melanoma (Balch CM *et al* 2001; Grande Sarpa H *et al* 2006; Balch CM *et al* 2009).

Once histology is available, informed decisions can be made as to the secondary stage of treatment. This will involve taking a specific wider excision margin, dependent on the tumour depth and other features. The skin defect is repaired accordingly, by direct closure, skin graft or more complex reconstruction of the defect, carried out by the local plastic surgeon.

Figure 1.10: Breslow Thickness

Depending on the Breslow depth of the melanoma, prognosis (5 year survival) can be approximated as follows:

<1mm – 95-100%

1-2mm – 80-96%

2.1-4mm – 60-75%

>4mm - 50%

These survival figures are from the AJCC guidelines (Balch CM *et al* 2001; Balch

CM *et al* 2009)
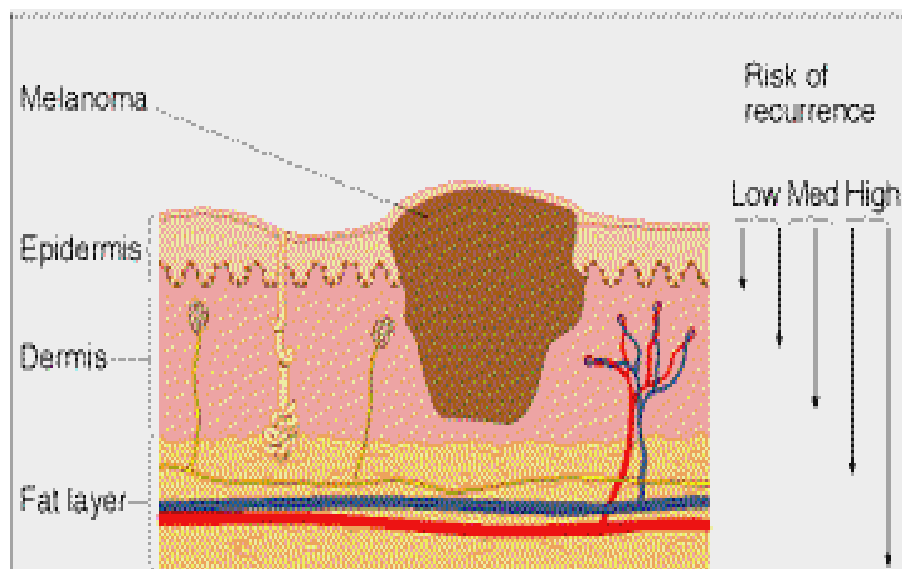
In patients who have clinically palpable lymph nodes, with histological evidence of melanoma, formal cancer staging is required. The introduction of sentinel lymph node biopsy has added another dimension to clinical and pathological staging. This is another important prognostic tool (Morton DL *et al* 2006), although there is a debate regarding its therapeutic efficacy (Balch CM and Cascinelli N 2006; Gonzalez U 2007; Balch CM *et al* 2009). If spread of disease is shown to be localised to one lymphatic basin, a block dissection of the involved lymph node basin is offered to the patient. The TNM staging system is used to give patients more complete prognostic information and to discuss further treatment options as necessary, once investigations and further surgery are complete.

The TNM Staging System was developed and is maintained by the AJCC. It was introduced as a tool for doctors to stage different types of cancer based on certain standard criteria. TNM is based on the extent of the tumour (T), the extent of spread to the lymph nodes (N) and the presence of metastasis (M). A new version of the 2002 TNM staging system for melanoma (Balch CM *et al* 2001; Balch CM *et al* 2009) has been recently published jointly by the AJCC and the International Union Against Cancer (UICC). The major change is the inclusion of mitotic activity as an important prognostic feature of the primary tumour. A mitotic rate of greater than $1/mm^2$ indicates a higher risk of metastasis. This change was based on outcomes of 60,000 patients (Balch CM *et al* 2009). **Table 1.3** demonstrates the TNM staging system for malignant melanoma, and **Table 1.4** demonstrates staging with the associated predicted survival rates for 10 years as per the latest AJCC guidelines.

Table 1.3: TNM Staging in Malignant Melanoma: taken from revised 2009 – AJCC/UICC guidelines (Balch *et al* 2009)

Stage 0: Melanoma in Situ (Clark level I), 99.9% 5 year survival		
Stage I/II: Invasive Melanoma, 85-95% 5 year survival		
T1a	< 1.0mm Breslow	Without ulceration, Clarks II/III
T1b	< 1.0mm Breslow	With ulceration / Clarks IV/V
T2a	1.0mm – 2.0mm Breslow	Without ulceration
Stage II: High risk Melanoma, 40-85% 5 year survival		
T2b	1.0mm – 2.0mm Breslow	With ulceration
T3a	2.0mm-4.0mm Breslow	Without ulceration
T3b	2.0mm-4.0mm Breslow	With ulceration
T4a	4.0mm or greater	Without ulceration
T4b	4.0mm or greater	With ulceration
Nx: Regional nodes cannot be assessed		
N0: No regional node metastasis		
Stage III: Regional metastasis, 25-60% 5 year survival		
N1	Single positive lymph node	A: micrometastasis B: macrometastasis/clinically apparent
N2	2-3 positive regional lymph nodes or intralymphatic regional metastasis	A: micrometastasis B: macrometastasis / clinically apparent C: Satellite or intransit metastasis without nodal mets
N3	4 or more metastatic nodes, or matted nodes or intransit metastases/satellites with metastatic nodes.	
M0: No evidence of metastasis to distant tissues or organs		
Stage **IV**: Distant Metastasis, 9-15% survival		
M1a	Distant skin, subcutaneous or metastases	Normal LDH
M1b	Lung metastases	Normal LDH
M1c	All other visceral metastases Or any distant metastases	Elevated LDH

Table 1.4: Melanoma staging and predicted 10 year survival (Balch *et al* 2009)

Stage	TNM	5 year survival	10 year survival
Stage IA	T1aN0M0	99%	97%
Stage IB	T1b/2aN0M0	92%	86%
Stage IIA	T2b/3aN0M0	78%	66%
Stage IIB	T3b/4aN0M0	68%	59%
Stage IIC	T4bN0M0	56%	48%
Stage IIIA	T1-4N1aM0 / T1-4aN2aM0	51-70%	46-65%
Stage IIIB	T1-4bN1/2aM0 / T1-4aN1/2bM0 / T1-4a/bN2cM0	50-68%	44-60%
Stage IIIC	T1-4bN1bM0 / T1-4bN2bMO / Any TN3M0	27-52%	22-37%
Stage IV	AnyT AnyN M1a-c	18%	14%

1.4.4 Treatment and clinical follow-up

As previously mentioned, current treatment involves primary surgical excision of the lesion in question. Once a lesion has been histologically confirmed to be a melanoma, wider margins are taken at operation, with the standard margin being 1cm of skin excised for every 1mm depth in Breslow thickness. Numerous studies have shown that there is no difference between 1 and 3 cm excision margins in patients with melanomas <2mm in depth, and also that 2cm excision margins are adequate for 1-4mm depth melanomas (Veronesi U and Cascinelli N1991; Balch CM *et al* 1993). A recent Cochrane review published in 2009 looked at all randomised controlled trials (RCTs) of surgical excision of melanoma comparing different width excision margins. Data was analysed looking at survival and recurrence, and found that recurrence free survival favoured wider excision margins, but that this was not statistically significant (Sladden M *et al* 2009).

All patients with melanoma are closely surveyed post operatively in the clinic and monitored closely for recurrence or development of distant metastatic disease for 5 years. If at follow-up, nodal or metastatic spread is suspected, then formal histological and radiological staging is performed.

1.4.5 The oncologists' challenge in melanoma treatment and novel therapeutic strategies

Patients with metastatic melanoma (stage IV) have a short median survival in the range of 6 to 9 months, with a 14% 10 year survival rate as shown in **table 1.4**.

Discouragingly, the median survival has not improved appreciably despite decades of investigations of cytotoxic, immunologic, and now molecularly targeted agents. It remains one of the most chemoradioresistant adult malignancies and treatment of advanced melanoma poses a huge challenge to the oncologist.

Single agent chemotherapy in melanoma produces objective response rates of less than 20%. Historically, dacarbazine (DTIC) has produced response rates of from 15% to 25% , but less than 5% of these are complete responses (Balch CM 1997). Long term follow-up of patients treated with DTIC alone shows that <2% can be anticipated to survive for 6 years. This remains the only cytotoxic drug approved by the U.S Food and Drug Administration (FDA) for the treatment of metastatic melanoma, despite its low single agent activity. A viable alternative to this agent is a closely related oral congener of DTIC, temozolomide, discussed in more detail in chapter 3. This has been established as a single agent in clinical trials, and shows similar efficacy, with the added advantages of its ability to penetrate the central nervous system, and therefore treat cerebral metastases, and its oral bioavailability, improving patient quality of life (Newlands ES *et al* 1992; Bleehen NM *et al* 1995; Middleton M *et al* 2000; Bafoloukos D *et al* 2002). More recently, the largest ever randomised, phase III trial conducted in stage IV melanoma, showed no survival benefit of using single agent temozolomide over DTIC (Patel PM *et al* 2008). Median overall survival reached 9.13 months with temozolomide and 9.36 months with DTIC. Median progression-free survival was similar and unimpressive at 2.30 months and 2.17 months, respectively. The principal investigator of this trial, Dr.Patel stated that 'the standard of care for these patients has not really changed in the last 2 decades' (Patel PM *et al* 2008).

Other single agents that have been tested in the treatment of metastatic melanoma include cisplatin and carboplatin. Cisplatin has induced a 15% response rate with a short median duration of 3 months (Atkins MB 1997; Glover D *et al* 2003). Also, the nitrosoureas - carmustine and lomustine - have been shown to induce objective responses in 13 to 18% of patients. Their advantage was thought to be their ability to traverse the blood-brain barrier, but despite these showing little or no activity against melanoma brain metastases, they have frequently been included in multi-agent regimens (Boaziz C *et al* 1991; Calabresi E *et al* 1991). The most interesting agent, however, in this group is fotemustine which has been directly compared in a phase III study with dacarbazine. This study showed that overall response rate (ORR) was higher in fotemustine arm compared with dacarbazine in first-line treatment of disseminated melanoma (Avril MF *et al* 2004).

The vinca alkaloids, particularly vindesine and vinblastine, have produced responses in approximately 14% of patients, and the taxanes in 16% of patients (Quagliana JM *et al* 1984; Einzig AI *et al* 1991; Aamdal S *et al* 1994). These agents are rarely used as single-agent therapy, but are frequently incorporated into combination chemotherapy or biochemotherapy regimens. More hopeful as a single agent is a potentially active new drug called abraxane (ABI-007), which is an albumin-bound nanoparticle formulation of paclitaxel. It has so far shown >30% overall response rate in phase II trials, and can now be explored in a randomised phase III trial (Hersh E *et al* 2006).

Since single-agent treatment has shown only modest response rates in the treatment of

metastatic melanoma, combination regimens have been put forward for clinical trials. The development of these combination regimens was based on the minimal single-agent activity seen, without any laboratory or clinical evidence of synergistic activity. Unsurprisingly, 2-agent regimens of the afore-mentioned compounds showed little or no superiority in response rates to single agent regimens i.e. 10 to 20% (Costanzi JJ *et al* 1975; Vorobiof DA *et al* 1986; Avril MF *et al* 1990). These disappointing trials were followed by phase II studies of 3 and 4 drug combinations, which generally produced response rates from 30 to 50%. However, when randomised studies were subsequently carried out to compare single agent DTIC to a triple agent regimen of cisplastin, vinblastine and DTIC (CVD), and also to quadruple agent therapy, there were no overall differences in either response rates or survival (Legha SS *et al* 1989; Rusthoven JJ *et al* 1996).

Further combination studies have subsequently been carried out, in the quest to achieve better response rates and improved survival rates in this seemingly resistant metastatic cancer. Both tamoxifen and interferon α have been used in combination with DTIC and in other multi-agent regimens. Smaller phase II and III trials have suggested significant benefit in response rates with these additional agents. However, these benefits have not been confirmed in large, multicentred Phase III trials, suggesting probable selection bias (Flaherty LE *et al* 1997; Lens MB *et al* 2003).

Biochemotherapy has also been evaluated in recent years, looking at the role of interferon α (IFNα) and interleukin 2 (IL2) in combination with traditional chemotherapy regimens. In a metaanalysis by Keilholz et al, patients with metastatic melanoma who were treated with IL-2/IFNα/chemotherapy, IL-2/IFNα without

chemotherapy and IL2/chemotherapy without IFNα had response rates of 45%, 21% and 15% respectively. However, there was no significant survival benefit for any of the regimens (Keilholz U *et al* 1998). Multiple biochemotherapy studies in differing regimens have been subsequently carried out, and the overall conclusion to date is that biochemotherapy should not be used outside of clinical trial in stage IV disease. It may be useful in the symptomatic patient with rapidly progressive disease.

As has been so far demonstrated, the treatment of metastatic melanoma remains challenging, and it remains intrinsically resistant to most types of chemotherapy and biological therapies. This, as is the case for the treatment of many adult tumours, has led investigators to evaluate new approaches and to assess newer molecularly targeted therapies.

There are many new agents currently under trial in the case of melanoma. Some of the more recent trials include agents such as protein-kinase inhibitors e.g. sorafenib, agents that act on cytotoxic T-lymphocyte antigens (CTLA-4 or ipilimumab) or on apoptotic mechanisms e.g. oblimersen sodium and specific anti-angiogenic agents e.g. bevacizumab, SU5416, MEDI-522, PI-88 and B-RAF inhibitors e.g plexikon (ref). Some of these agents and associated melanoma trials are detailed below:

Sorafenib targets the adenosine triphosphate-binding site of the B-RAF kinase. Melanoma exhibits 70% B-RAF mutation in comparison to 7% mutation in all other adult tumours, making sorafenib an ideal molecularly targeted melanoma therapy. It is a drug which not only inhibits B-RAF, but also tyrosine-kinases involved in angiogenesis and tumour progression. Results from phase I and II trials to date have

shown some promising results, both as a single agent and in combination with traditional chemotherapeutic agents. One phase II trial combined sorafenib at 3 different doses with carboplatin and paclitaxel, showing a 31% response rate, and 54% stable disease in a group of 35 patients with metastatic melanoma (Flaherty K *et al* 2005). It has also been combined with DTIC and compared with placebo showing a 51.3 versus 45.6 month overall survival benefit (McDermott D *et al* 2008).

Sorafenib and Temozolomide have been combined in the treatment of melanoma (Phase II). The selection of these agents has been based on melanoma-specific effects seen when both are given as single agents (Flaherty K *et al* 2005). Also, pre-clinical studies have demonstrated that Sorafenib enhances the therapeutic efficacy of a broad range of chemotherapeutic agents in xenograft models of various cancers (Queirolo P *et al* 2006). However, within the combination trial (Flaherty K *et al* 2005), the anti-angiogenic effects of the regimen was not assessed, with the end-point being progression-free survival. The combination showed encouraging activity in patients with metastatic melanoma, particularly in those with brain metastases.

However, results of a phase III randomised, placebo-controlled study of sorafenib in combination with carboplatin and paclitaxel as a second-line treatment for advanced melanoma showed that the addition of sorafenib did not improve any of the end-points (PFS and OS) (Hauschild A *et al* 2009).

Imatinib mesylate is an oral tyrosine kinase inhibitor of c-Kit, PDGFR and BCR-ABL. Expression of c-Kit (a transmembrane receptor with TK intracellular domain) was seen in nearly 50% of early melanomas, but this is reduced in the metastatic

phenotype (Janku F *et al* 2003). A phase II trial of Imatinib was performed in 18 patients with metastatic melanoma and at least 25% c-Kit expression. Only one patient had high expression of c-Kit (75%) and in this patient, a near complete response was observed. Unfortunately, all other patients progressed (Ugurel S *et al* 2005). A further phase II study showed similar results in a patient with a metastatic acral lentiginous melanoma and high c-Kit expression (Kim K *et al* 2008). There is some evidence to show that the very rare mucosal melanomas may have greater c-kit expressiom, and therefore may be more responsive to this agent.

Temsirolimus is a novel potent inhibitor of rapamycin (mTor). mTor is a serine-threonine kinase that promotes phosphorylation of S6K1 and the eukaryotic initiation factor 4E- binding protein-1 (4E-BP1), involved in G phase progression. Temsirolimus acts as a rapamycin analog, and subsequently blocks the phosphorylation cascade (Meric-Bernstam F and Mills G 2004). A phase II study of intravenous temsirolimus every week in 33 patients with metastatic melanoma was undertaken (Margolin K *et al* 2005). Toxicities were mild, but only one patient had a partial response, concluding that, as a single agent, it was not effective in melanoma.

Bevacizumab is a recombinant humanized monoclonal antibody designed to specifically bind VEGF (see section 1.2.4), and hence inhibit its biological activity, leading to reduced tumour microvasculature (Kerbel R and Folkman J 2002). VEGF is overexpressed in melanoma and its expression strongly correlates with poor prognosis (Ugurel S *et al* 2001). A phase II trial of Bevacizumab (15mg/kg i.v every 2 weeks) with or without daily subcutaneous interferon α was carried out. Out of 16 patients with metastatic melanoma, 1 complete response, 1 partial response and 4

disease stabilisations were seen in patients who were receiving both agents (Carson W *et al* 2003).

MEDI-522 is a humanized monoclonal antibody designed to target integrin alphavbeta3 (avb3). Avb3 has been linked to invasive growth and metastasis in melanoma, and also to up-regulation of bFGF (a pro-angiogenic factor). In a phase II trial of dacarbazine 1000mg/m^2 every 3 weeks plus MEDI-522 (55 patients) or just MEDI-522 alone (57 patients), a 13% response rate was seen in the combination arm, and promising improvement in survival (11.8 months) with single agent (Hersey P *et al* 2005).

PI-88 is a new anti-angiogenic agent. It mimics heparan sulphate, and competitively binds both angiogenic growth factors such as FGF and VEGF, inhibiting angiogenesis, and also heparanase, thus preventing degradation of the extracellular matrix (a major step in metastasis). In a phase II trial of PI-88, 250mg/day was given for 4 days every week to pre-treated patients. Disease control rate was 36% (Joyce J *et al* 2005; Thomson D *et al* 2005).

The expanding list of targeted agents and potential combination regimens continues to increase exponentially, but sadly those trialled to date fail to show any significant survival benefits to the traditional DTIC based regimens. This failure may be attributed to the empiricism of trials that generally combined agents because they were there, rather than a scientific understanding of mechanisms of action and proven synergy (Agarwala S 2008). Malignant melanoma progression is determined by several parallel and interacting pathways affecting growth control, differentiation, cell

adhesion, invasion, angiogenesis and resistance to apoptosis. Integration of the increasing understanding at the molecular level of the generic mechanisms governing melanoma spread, along with the evidence of the effects of the host immune system on melanoma, may finally produce a way forward in this intractable disease. In the meantime, the best service clinicians can provide patients with advanced, devastating melanoma is to encourage enrolment onto clinical trials.

1.5   Thesis Objectives

From a basic science perspective, the objectives of this thesis are to assess the anti-endothelial effects of cytotoxic agents given in a metronomic schedule as single agents and in combination with specific anti-angiogenic compounds. Optimization of the previously established technique of the HUVEC model will be a major component of the work (Lam T *et al* 2007). Along side this, to set up a scientific sub-study, as part of an existing phase I/II clinical trial of combined cytotoxic chemotherapeutic agents with a non-specific anti-angiogenic compound. This trial was using a metronomic dosing schedule in patients with metastatic malignant melanoma. Amongst the trial group, the aim was to assess the profile of circulating endothelial cells and circulating endothelial progenitor cells – potential biomarkers of response to anti-angiogenic therapy (see section 1.2.3 and 1.2.4). Comparison of this biomarker will be made between the traditional high-dose group and the metronomically dosed group of patients.

Chapter 2

Materials and Methods

This chapter includes two methodologies, the first in relation to the *in vitro* MVEC model experiments (section 2.1), and the second in relation to the clinical study (section 2.2) – isolation of CECs and CEPs in patients with metastatic melanoma. The optimisation of the technique used for the clinical study will be discussed along with the clinical results in chapter 5.

2.1 The *in vitro* MVEC model

The main aim of this part of the study was to investigate potential anti-endothelial properties of several orally bioavailable cytotoxic agents when given in a continuous low dosing schedule. These agents were assessed as single agents and in combination with specific anti-angiogenic agents i.e. sorafenib and combretastatin. The study used microvascular endothelial cell proliferation as a surrogate marker of *in vitro* anti-endothelial response (see section 1.2.3). The study of angiogenesis has been significantly advanced by the ability to culture endothelial cells *in vitro,* recognising the limitations of *in vitro* models. This was initially introduced using large vessel endothelial cells, such as those isolated from the human umbilical vein (HUVECs). However, through previous work within this study group and from other researchers (Bocci G *et al* 2002; Lam T *et al* 2007) MVECs have been identified as a more suitable model to study anti-endothelial effects, and are VEGFR-2 positive (Bouis D *et al* 2001). Endothelial cells are heterogenous in phenotype, function, expression of surface molecules and responsiveness to growth factors. MVECs comprise only 1-5% of cells in a given tissue, grow slowly and are contact inhibited. The process of

neovascularisation involves recruitment of microvascular rather than large endothelial cells. MVEC expression of actin binding LIM protein 1, myosin 5C and myosin 7A relates to their ability to undergo extensive cytoskeletal remodelling and migration during angiogenesis (Manconi F *et al* 2000; Giusti B *et al* 2006). These cells are however very delicate, and require fastidious culture techniques, described in **section 2.1.1.2**.

For this study, MVECs were grown in monolayers and a protocol was then followed which allowed protracted drug exposure, guided by previous studies (Bocci G *et al* 2002; Lam T *et al* 2007). In applying this metronomic principle to the *in vitro* setting, one is limited by the time at which cells reach confluence i.e. there is a finite duration of treatment. It was therefore necessary to perform optimisation experiments to determine the 'optimum' duration of cell growth *in vitro*.

Once optimum duration of cell growth was determined, the chemosensitivity experiments were performed. Cell proliferation was the main parameter assayed, using growth inhibition as a marker of anti-endothelial efficacy, and to determine the presence or absence of a synergy in the combined schedules. The assays were conducted on human tumour cell lines for comparative purposes and, in the case of the melanoma cell line, to assess any additional anti-melanoma effect.

2.1.1 General cell culture

All media and tissue culture reagents used were purchased from Invitrogen (Paisley, UK) unless otherwise stated. Plastic ware was purchased from Sarstedt, UK unless otherwise stated. All media and reagents mentioned in this chapter are listed and described in **Appendix 1**.

2.1.1.1 Aseptic technique

Aseptic techniques were conducted in a Class II microbiological safety cabinet (ICN flow) fitted with a UV sterilising lamp. All glass ware and heat-stable solutions were auto-claved prior to use at $121^{o}C$ for 10 minutes.

2.1.1.2 Maintenance of primary cells and cell lines

Human dermal microvascular endothelial cells (MVECs) were purchased from TCS CellWorks (UK) (no. ZHK-2526). Human melanoma cell line SK-MEL-2 and human breast carcinoma cell line MCF-7 were purchased from the American Type Culture Collection, and the human lung carcinoma cell line A549 was purchased from the European Collection of Cell Cultures. The three human cancer cell lines were utilised as controls to assess the anti-cancer effects of each agent.

MVECs were maintained in Medium 199 (no. M4530, Sigma-Aldrich) supplemented with MVEC Growth Supplement (no. ZHS-8947 - TCS) + antibiotics (no. ZHR-9939 - TCS) – pre-equilibrated in a $37^{o}C$, CO_2 incubator prior to culture of cells.
SK-MEL-2, MCF-7 and A549 cells lines, as described above, were all maintained in medium plus supplements (see **Appendix 1**). Maintenance of all cells was in 75cm^2 tissue culture flasks, each flask containing 15ml of the relevant supplemented media. This was in a humidified atmosphere of 5% CO2 at $37^{o}C$.

All cells were used at passage 6 or below, as it is recognised that morphology and receptor status can alter with increasing passage. MVECs were harvested with a solution of 0.025% (w/v) trypsin / 0.01% (w/v) EDTA as recommended and provided by TCS CellWorks Ltd, U.K. The SK-MEL-2, MCF-7 and A549 cells were harvested

with TrypLE™ Select (Invitrogen), which has enhanced activity on strongly adherent cell lines (see **Appendix 1** for further details). Passage of cells was carried out when growth was in a logarithmic phase, at 70-80% confluence. This was done by the addition of 5ml of the appropriate trypsin solution to each flask, which was then incubated at 37^0C until 90% detached and for no longer than 5 minutes. Detachment was increased by a gentle tap when examining flasks at intervals between 3 to 5 minutes under the microscope. Once detached, an equivalent amount of media was added to inactivate the trypsin and the cells recovered by centrifugation at 220g for 3 minutes. The cells were then passaged into fresh $75cm^2$ flasks (usually at a ratio of 1:3), or frozen down in 1ml aliquots as required and depending on the passage. A confluent flask was split into 4x1ml aliquots for freezing down, at a concentration of $0.5-1.0x10^6$ cells/ml (see 2.1.1.4 for cell counting).

For MVECs, prior to passage, flasks were first incubated with 5ml attachment factor (see **Appendix 1**) per $75cm^2$ flask for 1 hour. The excess was then aspirated off and the appropriate amount of cell suspension added to MVEC media. This ensured cell adherence as recommended by TCS cell works.

2.1.1.3 Cell freezing and recovery

In order to freeze samples, harvested cells, using steps described in section 2.1.1.3, were instead resuspended in freeze medium (see Appendix 1) at a concentration of $0.5-1.0x10^6$ cells/ml (see section 2.1.1.5 for cell counting). 1ml aliquots were then transferred to cryovials. The cryovials were kept in a -80^oC freezer for up to 24 hours, and then transferred to liquid nitrogen for long term storage.

For recovery, cells were rapidly thawed by placing a frozen aliquot into a 37°C water bath for 1-2 minutes until fully thawed and the cell suspension was slowly added into a pre-warmed (37°C) culture medium – specific to each cell line (see Appendix 1) - and centrifuged at 220g for 3 minutes. The pellet was then resuspended in approximately 15ml of fresh medium and placed in a 75cm^2 tissue culture flask.

2.1.1.4 Cell counting

To determine the concentration of cells a haemocytometer was used. Cell suspension (25μl) was diluted 1:1 with 0.1% (w/v) Trypan blue stain. Trypan blue is a vital dye that is excluded by viable cells. Non-viable cells show cytoplasmic staining due to Trypan blue uptake. This diluted suspension was well mixed, and placed into a haemocytometer chamber. The cell density was determined under the light microscope by enumerating the number of cells in a set area i.e. 5 large squares, and gaining an average of this count (divide by 5). The following formula was then used:

$$\text{Cell concentration (cells/ml)} = \text{cell count x 2 (dilution factor) x } 10^4$$

2.1.1.5 Optimisation and determining seeding densities

Preliminary experiments were conducted to determine the optimal cell seeding density which enabled the longest period of uninterrupted growth in 96-well plates up to confluence. This was carried out by plating out all four cell types on day 1 at varying densities of 1000 to 5000 cells per well in 200μL of cell specific media. The specific media, attachment factor (for MVECs) and growing conditions remained the same for each cell type as previously mentioned. All cells were kept in a humidified atmosphere of 5% CO_2 at 37°C. Confluence for each cell type and each seeding density was then assessed on each day, and expressed as an approximate percentage.

200μL of the appropriate cell-specific media was removed and replaced daily for each well and the experiment was carried out in triplicate for all cell types. **Figure 2.1** shows a summary of the method used to plate out for this assay.

2.1.2 Chemosensitivity assay

2.1.2.1 Drugs

Purified temozolomide was provided by Schering-Plough, U.K. Purified Paclitaxel (no. T7402), etoposide (no. E1383), vinorelbine (no. V2264) and carboplatin (no. C2538) were from Sigma-Aldrich, UK. Purified sorafenib tosylat was provided by Bayer, U.S.A. Purified Combretastatin was provided by Oxigene, UK. Purified Bortezomib was provided by Millenium, UK. For *in vitro* experiments, drugs were reconstituted following manufacturers instructions at 10mM in DMSO, except for Vinorelbine which was reconstituted at 10mM in sterile water, and Combretastatin which was reconstituted at 10mM in PBS. The 10mM solutions were then diluted in culture medium immediately prior to use, into appropriate aliquots at the specific concentration required. The concentration of DMSO in culture was kept at 0.1% (v/v), which therefore acted as control in all experiments. The effect of DMSO at varying concentrations was first assessed in all cell types to ensure that the chosen concentration of 0.1% did not cause any effect independent to the agents being tested.

2.1.2.2 Treatment of cells with single agents and with combinations of cytotoxic and anti-angiogenic agents

In vitro chemosensitivity testing was performed on cells grown as a monolayer on 96-well flat-bottomed tissue culture plates (Sarstedt). Each drug concentration was set

Figure 2.1: Summary diagram of plate set-up for the chemosensitivity assays

On days 2,3 and 4, the dosing schedule for each drug was undertaken on the plate for each of the four cell types. The concentration of DMSO was kept at 0.1% throughout.

Media replaced daily

↑   drug concentration

First column of cells dosed with media containing 0.1% DMSO
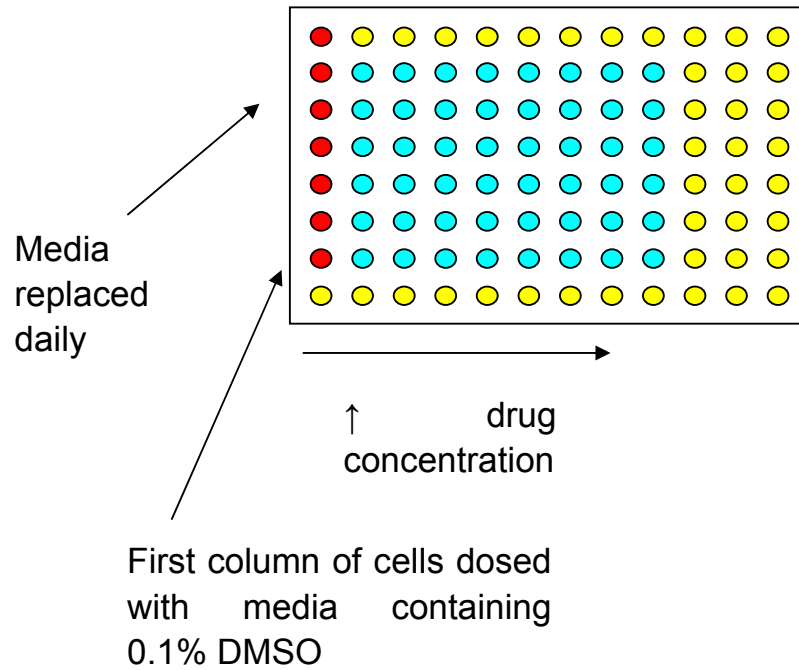
Key: **Red** – cells dosed with 0.1% DMSO and media daily i.e. column 1

Blue – cells dosed with increasing concentrations of drug i.e. column 2-9, in sextuplicate.

Yellow – media only

up in sextuplicate. Preliminary experiments were then undertaken to determine the therapeutic range of each drug. Cells were treated with Temozolomide (0-100μM), Paclitaxel (0-2nM), Vinorelbine (0-10μM), Etoposide (0-5μM), Carboplatin (0-500μM), Sorafenib (0-10μM), Combretastatin (0-25nM) and Bortezomib (0-100nM) as single agents. For all experiments, in order to maintain constant concentration of the drugs, the media was replenished with fresh solutions containing the appropriate drug concentrations on a daily basis 4 days, as determined by preliminary experiments to determine the duration of drug exposure for all cells. (Bocci G *et al* 2002; Lam T *et al* 2007).

Initial dose ranges and drug concentrations were based on pharmacokinetic data from phase I clinical trials. For the five traditional cytotoxics, the clinical maximum tolerated dose (MTD) from these original studies, was converted into a molar concentration (see **table 2.1**) (Koeller J *et al* 1986; Sessa C *et al* 1995; Brada M *et al* 1999; Malingre M *et al* 2000; Marty M *et al* 2001). Since 'metronomic dosing' is traditionally devised upon an empirical dose between 10-33% of the MTD (Maraveyas A *et al* 2005; Lam T *et al* 2006), a wide *in vitro* dose range was chosen from 0 to 50% of the MTD for each drug. This choice of *in vitro* dose ranging was similarly used by Lam et al (Lam T *et al* 2007) and in the study based on the HUVEC model which used by Bocci and colleagues (Bocci G *et al* 2002). For the specific anti-angiogenic agent – sorafenib - the actual clinical dose – 400 mg twice daily, was converted into a molar concentration from Phase I data, equivalent to 4.5μM (Moore M *et al* 2005) and a wide dose range selected to include this in order to demonstrate anti-endothelial and potential anti-proliferative effects. For the non-specific anti-angiogenic agents - combretastatin and bortezomib – a wide dose range was also

Table 2.1: **Published phase I pharmacokinetic data for oral formulations of the**

drugs used in this study

Drug	Published MTD (mg/m^2)	Published Cmax for MTD	Reference	Equiv. molar conc. of Cmax (μM)	*In vitro* dose range selected (μM)
Temozolomide (oral) mol wt 194.15	200	13.9μg/ml	(Brada M 1999)	71.5	0-50
Paclitaxel (oral)	300	0.33μM	(Malingre M 2000)	0.33	0-0.002
Vinorelbine (oral) mol wt 1079.2	80	133.4ng/ml	(Marty M 2001)	0.123	0-10
Etoposide (oral) mol wt 588.56	220	21.1μg/ml	(Sessa C 1995)	4.5	0-10
Carboplatin (oral)	1000	38.5μM	(Gore M 1987)	38.5	0-500
The Cmax data at the MTD was converted into an equivalent m concentration and this was used to select an in vitro dose range. Refere refer to both published MTD and Cmax for MTD.					

selected, guided by previous in vitro and in vivo studies (Stevenson J *et al* 2003; Papandreou C *et al* 2004; Schwartz R *et al* 2004).

2.1.2.3 Cell proliferation assay (MTT)

To determine the effects of chemotherapy on cell proliferation, a standard MTT (see Appendix 1) assay was performed (no. M5655, Sigma-Aldrich,UK). The optimal cell seeding density for each cell type was used, and cells resuspended in 200μL of appropriate media (attachment factor was used to prepare plates for MVECs as described in 2.1.1.2-20μL per well). Plates were then incubated for 24 hours at 37^0C in a humidified atmosphere of 5% CO_2 to allow the cells to adhere to the well surfaces. The cells were divided into two groups, one used as a control in order to determine cell number at start of treatment (baseline). The other group was the treatment group. The MTT assay was therefore performed immediately prior to drug addition in the control group. It was then performed at the end of the period of drug treatment (96 hours), and dose-response curves. 20μL of MTT solution was added to each well containing 200μL of cell-specific media, and the mixture incubated at 37^oC for 4 hours. The substrate medium was then removed; 200μL of lysis buffer (see Appendix 1) was then added to each well and plates were incubated for a further 2 hours. Optical density at 570nm was then read using a spectrophotometric plate reader.

2.1.2.4 Calculation of the relative growth

Using results produced from the spectrophotometric plate reader, cell growth from baseline was expressed as percentage change in absorbance values. To calculate the relative growth, the growth of control (untreated) cells was taken as 100%, and the

corresponding growth of treated populations of cells was expressed as a percentage of control growth.

2.1.3 Statistical analysis of MTT data

All data represent the mean of three independent experiments and each drug concentration was set up in sextuplicate. The results of relative cell growth are expressed as mean values +/- standard error. The data was analysed using a two-sample t-test for populations of unequal variance to compare the effects of different drug doses and combinations. SPSS software version 14.0 (SPSS, Chicago, USA) was used for the statistical analysis. A confidence level of $P < 0.05$ was considered statistically significant.

2.2 Isolation of circulating endothelial cells and circulating endothelial progenitor cells in patients with metastatic malignant melanoma

2.2.1 Clinical study protocol and participant recruitment

Before commencing the study, ethical approval was granted by the local research and ethics committee (06/Q1105/38) and by the Hull and East Yorkshire NHS Trust (R0397). All forms including consent and information sheets to be completed by study participants were also approved (see Appendix 2).

The clinical study involved recruitment of participants from an existing trial entitled 'A Phase I/II study of Estramustine and Temozolomide in patients with metastatic malignant melanoma'. This trial is offered to patients with advanced malignant

melanoma, and had already been approved by the Hull and East Riding LREC in 2002 (05/Q1105/76). This trial was set up to determine the metronomic dose of temozolomide and estramustine in this patient group, based on previously articulated dosing principles (Lam T *et al* 2006; Lam T *et al* 2007) The ultimate aim is to add in a specific anti-angiogenic agent as a third agent once the metronomic dose was reached. The metronomic, combinatorial approach being used in this trial is therefore closely linked to the theory behind the *in vitro* MVEC model chemosensitivity assay as discussed earlier in this chapter (section 2.1).

Practice concurrent with the period of the experimental work of this thesis was to treat patients with conventional DTIC based chemotherapy based on MTD or to offer patients' entry in to the temozolomide-estramustine Phase I trial. This trial has two dosing schemes - one is that of MTD while the other is establishing a continuous daily dosing schedule, a 'metronomic-like' approach, using a temozolomide-based dose-escalation schema (**Table 2.2**). Patients from each of these treatment sub-groups were included in this thesis study, being separated in to patients treated with the MTD (conventional) schedule and patients treated with 'metronomic-like' schedule. . The study can therefore be classed as a scientific sub−study to the already existing treatment trial, and had no influence on patient management. Patients had the opportunity to discuss the study in detail in the clinic. The GCP consent process was followed. The patients were asked to read through a patient information leaflet, either at the clinic or in their own time (see Appendix 2.1). Only once they had had the opportunity to ask questions and read the information leaflet, were they then asked if they were willing to participate, and if so, then sign the consent form (see Appendix 2.2).

Table 2.2: Dose escalation schema for A Phase I/II Study of Estramustine and Temozolomide in Patients with Metastatic Melanoma.

The patients studied in the 'metronomic' arm of the study (described further in chapter 5) were on dose levels 5a and 6. As per metronomic principles, (Maraveyas A *et al* 2005; Lam T *et al* 2007) bone marrow toxicity was not seen in levels 5a and 6.

	Temodal schedule	Temozolomide	Estracyt schedule	Estramustine
Level -1	QD Days 1-7,15-21	75 mg/m^2	BD/TDS daily cnt	7.5 mg/kg
Level 1	QD Days 1-7,15-21	100 mg/m^2	BD/TDS daily cnt	7.5 mg/kg
Level 2	QD Days 1-7,15-21	100 mg/m^2	BD/TDS daily cnt	10 mg/kg
Level 3	QD Days 1-7,15-21	100 mg/m^2	BD/TDS daily cnt	12.5 mg/kg
Level 4 (started June 06)	QD Days 1-7,15-21	125 mg/m^2	BD/TDS daily cnt	7.5 mg/kg
Level 5	QD Days 1-7,15-21	125 mg/m^2	BD/TDS daily cnt	10 mg/kg
Level 5a	QD daily	75 mg/m^2	BD/TDS daily cnt	10 mg/kg
Level 6	QD Days 1-7,15-21	125 mg/m^2	BD/TDS daily cnt	12.5 mg/kg
Level 7	QD Days 1-7,15-21	125 mg/m^2	BD/TDS daily cnt	15 mg/kg
Level 8	QD Days 1-7,15-21	150 mg/m^2	BD/TDS daily cnt	7.5 mg/kg
Level 9	QD Days 1-7,15-21	150 mg/m^2	BD/TDS daily cnt	10 mg/kg
Level 10	QD Days 1-7,15-21	150 mg/m^2	BD/TDS daily cnt	12.5 mg/kg

For those patients who had given fully informed consent and wished to take part in this scientific sub-study, a 50 ml blood sample was taken before, during and after (0, 6-12 and 24-48 weeks) the onset of their treatment regimen, whether conventionally-dosed or on the metronomic schedule. Blood samples were taken at the same time as routine blood tests, so that no extra needle-stick was incurred. If patients chose not to be part of this sub-study, it had no influence on their treatment options or management.

Four healthy volunteers - age and gender matched - acted as the control group. These were patients attending a routine benign skin lesion clinic within the same hospital trust. They were asked to participate in a similar manner to the patient group. They were also given a relevant information leaflet (see Appendix 2.3) detailing the proposed study to read in their own time. If they then wished to participate, they were asked to sign a consent form (see Appendix 2.4). One 50 ml sample of the volunteer's blood was required, and then processed in the same way as the patient samples.

All blood samples were coded anonymously before transfer to the laboratory. All patient and healthy volunteer details were maintained in the coded form and stored on a password protected computer.

Patient Enrolment

Inclusion Criteria – each patient had to meet all these to be considered for enrolment in the chemotherapy trial, and therefore in the scientific sub-study:

- Malignant Melanoma AJCC stage IV

- Clinically staged and deemed suitable for a combined regimen of temozolomide and estramustine.

- Able to give written consent

Exclusion Criteria - a patient was not enrolled to either study if any of these criteria applied:

- Tumour other than melanoma

- Pregnant or lactating patients.

- Patients with reproductive potential not taking adequate contraception.

- Concurrent administration of other experimental or investigational agents.

- Other illnesses that may have effects on progenitor cells (e.g. Lymphoma, autoimmune diseases etc).

- Patients who had been previously exposed to any of the trial treatement regimens.

2.2.2 Isolation of CEPs and CECs using MACS® Technology

2.2.2.1 Preparation of PBMCs

The isolation of CEPs and CECs from peripheral blood is a process which involves numerous steps. The first few steps were carried out in order to isolate the peripheral blood mononuclear cells from the fresh blood samples. Following this, MACS® Technology (Miltenyi Biotech) was used, which has become one of the standard methods worldwide for cell separation. When performing MACS® separations from human blood samples, whole blood as well as pre-enriched peripheral blood mononuclear cells (PBMCs) can be used.

2.2.2.2 Sample preparation from fresh whole blood

Firstly, 50 mls of freshly drawn blood was taken from a consented participant using a pre-heparinised 50ml syringe under aseptic technique. Each syringe contained 1ml of heparin-sodium solution (1000U per mL heparin with a pH of 7.5). The sample was then taken to the laboratory for processing, which was carried out within a 2 hour window. During this time, samples were gently agitated to minimise risk of blood clotting or separating. Samples were then diluted with 2 times the volume of phosphate buffered saline (pH 7.4) (PBS- see Appendix 1).

2.2.2.3 Density gradient centrifugation

Each diluted whole-blood sample was then divided into 35 ml quantitites, and layered over 15ml of Histopaque (Sigma) in a 50ml tube and centrifuged at 400g for 30 minutes at 4^0c in a swinging-bucket rotor (without brake). The middle layer was then aspirated, taking particular care to aspirate the mononuclear cell layer at the interphase between the top layer of serum and the bottom layer of red cells (See **Figure 2.2**). The interphase cells (lymphocytes, monocytes and thrombocytes) were then transferred to a new 50ml tube, and washed again in PBS. This tube was then centrifuged at 300g for 10 minutes. The supernatant was then carefully removed completely. In order to remove platelets, the cell pellet was then resuspended in 50 ml of PBS and centrifuged again at 200g for a further 10 min. Most platelets remain in the supernatant upon centrifugation at 200g. The supernatant was then removed again, and a cell count performed, having resuspended the cells in 1ml of peripheral blood mononuclear cell (PBMC) freeze media (see Appendix 1).

Figure 2.2: Density Gradient Centrifugation for the isolation of PBMCs
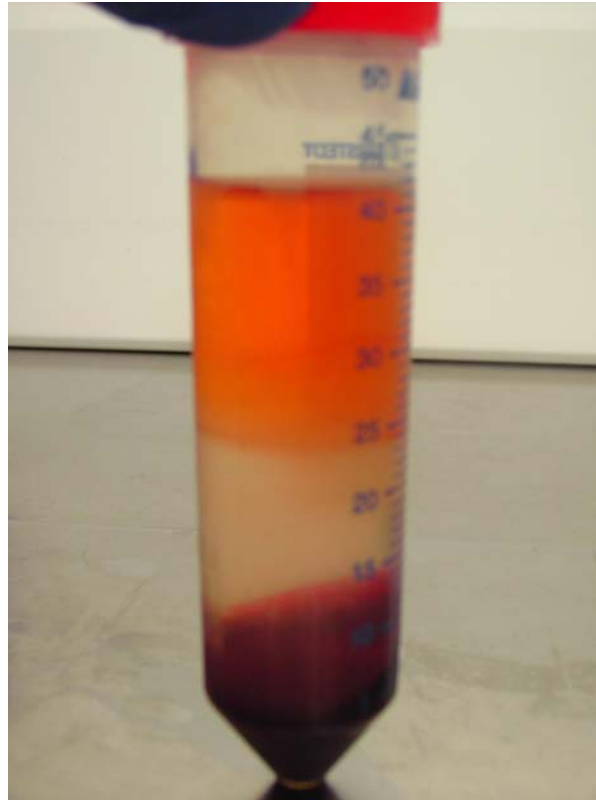
Photograph taken in laboratory during processing of whole blood samples

2.2.2.4 Sample storage

Freezing

Cryovials were labelled with the appropriate sample code, date and cell number information. Freezing medium (Appendix 1) was prepared from 90% fetal bovine serum and 10% dimethyl sulfoxide (DMSO), and then chilled on ice. Cells were then resuspended in freezing media at 1-1.5 $\times 10^7$ cells/ml (using same cell counting technique as in section 2.1.1.4) and transferred to the appropriate number of cryovials per sample (usually 2-3). The vials were then placed in the Nalgene 'Mr Frosty'(as described below) which was pre-chilled in the refrigerator at 4^0C. Once the samples were transferred to the 'Mr Frosty', they were then transferred into a liquid nitrogen cell storage system 24 hours later.

The Nalgene 'Mr Frosty' is a cryo 1^oC freezing container. It has an internal high-density polyethylene vial holder and foam insert within a polycarbonate unit. It is prepared by removing the holder and insert, and adding 250ml of 100% isopropyl alcohol to fill up to a pre-marked level which ensures that the vial holder will be surrounded by the isopropyl. The foam-insert and vial holder are then replaced, and the unit cooled in the refrigerator prior to the prepared vials being placed into holes in the vial holder. The whole unit is then placed in the -80^0C freezer, and allows a slow, even freezing process to take place, which aids in limiting cell damage and ice-crystal formation.

Thawing

The cryovial due to be processed was transferred from the liquid nitrogen into a 37^0C

water bath. It was held in the water bath, with the occasional 'flick' until a small amount of ice remained. The cryovial was then dried on its exterior to avoid contamination, prior to being transferred to the tissue culture. The cryovial contents were then transferred into a 15mL tube, and thaw media (90% RPMI + 10% FBS at 4^0C) was then added drop wise to the cell suspension. Having added 1mL, further thaw media was added slowly, first 2mL, then 4mL. This was then centrifuged at 250g for 10 minutes, and a further wash was then carried out – again using the thaw media. A cell count was then carried out before proceeding to the next step of magnetic labelling.

2.2.2.5 MACS® Technology – cell separation

MACS® Technology has become the standard method for cell separation, with numerous publications proving its versatility for multiple applications, from the laboratory bench to clinical applications, from frequently occurring cells to rare subsets (Jarrossay D *et al* 2001; Raia V *et al* 2007; Strijbos M *et al* 2008). The technology is based on MACS® Microbeads, MACS® Separators, and MACS® columns. MACS® microbeads are supermagnetic particles of approximately 50 nanometers in diameter. They are composed of a biodegradable matrix made up of iron oxide and polysaccharide, and therefore it is not necessary to remove them from cells after cell separation as they typically degrade after a few days in culture. They do not alter structure, function or activity status of cells (Kronick P and Gilpin R 1986; Miltenyi S *et al* 1990). MACS® Columns are the site of cell separation. Columns are placed in a MACS® Separator which is a strong permanent magnet. This creates a high-gradient magnetic field which is induced on the matrix of the column, strong enough to retain cells with minimal amounts of MACS® Microbeads. Cells

without microbeads attached (unlabelled cells) pass through the column and can be collected; cells with microbeads attached (labelled cells) are released after removal of the column from the magnet. Therefore, both labelled and unlabelled fractions can be easily isolated with a high level of purity (see **Figure 2.3**).

MACS® Technology allows a number of different cell separation strategies, and in this case both 'positive selection' and 'depletion' strategies are employed. 'Positive selection' means that the desired target cells are magnetically labelled and isolated as the magnetically retained cell fraction within the column. This strategy gives a high level of purity, especially for rare cell enrichment. 'Depletion' or 'untouched isolation' is performed by depleting the undesired cells. Non-target cells are magnetically labelled and eliminated from the cell mixture. The non-magnetic, untouched cell fraction which passes through the column contains the target cells (Safarik I and Safarikova M 1999). This is used for removal of unwanted cells; if no specific antibody is available for the target cells or for subsequent isolation of a cell subset by means of positive selection.

In this case, depletion was followed by positive selection, whereby the non-target cells are removed initially from the sample, and then the cell subset of interest is positively selected for. This is very useful as it depletes the non-target cells, leaving a pre-enriched fraction from which a pure cell sample can be eluted.

2.2.2.6 Depletion stage with CD45 MACS® MicroBeads

The application of CD45 Microbeads has been described (Meye A 2002) for enrichment of tumour cells from peripheral blood by the depletion of CD45$^+$

Figure 2.3: MACS® Microbead Separation
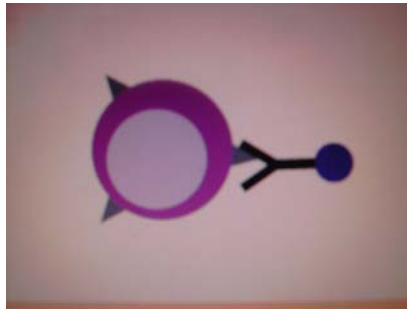
Antibody-microbead targets surface marker of specific cell – direct magnetic cell labelling

<u>Magnetic separation:</u>

The column is placed in the magnet, rinsed with buffer and labelled cells passed through. The effluent of unlabelled cells is collected, the column washed again with buffer, effluent is centrifuged and then a cell count is performed.

leucocytes. The CD45 antigen is expressed on all cells of haematopoietic origin except erythrocytes, platelets and their precursors. CD45 MicroBeads (cat #130-045-801 Miltenyi Biotec) were used in this case for depletion of leucocytes from peripheral blood. Reagents required for this stage included MACS buffer (see Appendix 1). This was kept cold at all times between 2-8°C.

The steps were carried out quickly, using pre-cooled solutions. This helps to prevent capping of antibodies on the cell surface and non-specific cell labelling. Capping has a number of proposed mechanisms, but essentially occurs on motile cells and is energy-dependant. It is a feature of molecules that are crosslinked, such as antibodies. If antibodies and cells are bound when cool (2-8^{0}C), patchy groups of antigen-antibody complex form around the cell. However, if warmed, they move to the back of the cell to form a 'cap'. Pre-cooling solutions aid in preventing this. Volumes given for magnetic labelling apply to samples up to 10^7 cells. When working with higher cell numbers, reagent volumes were scaled up e.g. 2×10^7 total cells required double reagent volumes.

Labelling

Firstly, for each sample the cell number was determined using the same cell counting techniques as previously described (section 2.1.1.4). The cell sample was then placed in the centrifuge and spun at 300g for 10 minutes. The supernatant was then aspirated completely and the cell pellet resuspended in 80μL of MACS buffer in a microcentrifuge tube. For every 10^7 cells present in the tube, 20μL of CD45 MicroBeads were added. The sample was then mixed well and incubated for 10 minutes at 4^{0}c. The cells were then washed with 2mLs of MACS buffer (see Appendix 1) per 10^7 cells and centrifuged again at 300g for 10 minutes. The

supernatant was then aspirated completely and the pellet resuspended in 500μL of MACS buffer for any cell number up to 10^8.

Magnetic Separation

The LD column (cat # 130-042-901) was placed in the magnetic field of the MACS® Separator, and rinsed with 2mL of MACS buffer. The labelled cells were then passed through the column, and the effluent of unlabelled cells collected. The LD column was then washed with 2 x 1mL of MACS buffer, only adding the second 1mL of MACS buffer once the column reservoir was empty. The total effluent was then centrifuged at 300g for 10 minutes, the supernatant aspirated and the cell pellet resuspended in 300μL of buffer for up to 10^8 cells. At this point, a further cell count was carried out (see **figure 2.4** for schematic).

2.2.2.7 Positive selection with CD34 MACS® MicroBeads

CD34 MACS® MicroBeads are used for identification and enumeration of CD34$^+$ cells by flow cytometry or fluorescence microscopy. They are useful in studies of phenotyping haematopoietic stem cells and haematologic malignancies, but also in the study of non-haematopoietic stem cells and in this case, CECs and CEPs (Peichev M *et al* 2000; Rafii S *et al* 2002). The CD34 antigen is a single chain transmembrane glycoprotein, expressed on human haematopoietic stem and progenitor cells, enodothelial progenitor cells, vascular endothelial cells, embryonic fibroblasts and some cells in fetal and adult nervous tissue (de Wynter EA *et al* 1998). MACS buffer was used for all steps in the labelling and separation stages of this process. The buffer was made up in the same way as described in Appendix 1, and kept cold at all times between 2-8°c, working quickly through each of the stages.

Labelling

The cell number for this step was determined as per previously described cell counting technique (section 2.1.1.4) at the end of magnetic separation with CD45 MicroBeads (cat #130-045-801 Miltenyi Biotec). This was followed by addition of 100µL of FcR blocking reagent (see Appendix 1) and 100µL of CD34 MicroBeads (cat #130-046-702 Miltenyi Biotec) to the sample, for any cell number up to a maximum of 10^8 cells. Incubation with FcR blocking reagent increases the specificity of antibody or MicroBead labelling, thereby improving the purity of target cells – in this case, endothelial cells. The sample was then incubated at 4°C for 30 minutes at 2-8°c. The cells were then washed by adding 5-10mL of MACS buffer for up to 10^8 cells and centrifuged at 300g for 10 minutes. The supernatant was then aspirated completely and the pellet resuspended in 500µL of MACS buffer.

Magnetic Separation

The MS column (cat #130-042-201 Miltenyi Biotec) was then placed in the magnetic field, and prepared by rinsing through with 500µL of MACS buffer. The labelled cell suspension was then applied onto the column, and the unlabelled cells collected (depleted of CD34$^+$ cells). The column was then washed with 3x500µL of MACS buffer, only adding new buffer when the column reservoir was empty. The total effluent was then collected, being the unlabelled cell fraction. The column was then removed from the magnetic field and placed over a 15mL tube. 1mL of MACS buffer was then added to the column and the positively-selected CD34$^+$ fraction was immediately flushed out by pushing the plunger into the column. Then the sample was washed with 10mL of MACS buffer, and spun at 300g for 10 minutes in the centrifuge. The spun sample was then resuspended in 100µL of MACS buffer and divided into two in order to generate a negative control for each sample (**figure 2.4**).

Figure 2.4: Schematic flow diagram to show steps of depletion followed by positive selection (MACS Technology)

1st magnetic labeling
Non-target cells are magnetically labeled with a biotinylated antibody cocktail and Anti-Biotin MicroBeads.

1st magnetic separation
Undesired cells are retained in a MACS® Column placed in a MACS Separator while the unlabeled cells pass through.

2nd magnetic labeling
Target cells are magnetically labeled with MicroBeads according to a subset marker.

2nd magnetic separation
Target cells are retained in the column while unlabeled cells pass through.

After the column is removed from the separator, the target cells are eluted as the enriched, positively selected cell fraction.

Undesired cells – CD45 positive, are retained in column. Unlabelled fraction containing target cells is then labelled with CD34 and target cells and positively selected.

2.2.2.8 Immunofluorescent staining with MACS$^{®}$ CD34-FITC and CD133/2 (293C3) antibodies

The CD34-FITC (cat #130-081-001 Miltenyi Biotec) in this case was used for identification and enumeration of CD34^{+} cells (CECs and CEPs) by flow cytometry. Identification occurs since the CD34-FITC is composed of monoclonal antibodies (Clone AC136) which recognise a class III epitope of the CD34 antigen with high specificity. Antibodies are conjugated to the FITC tag and are supplied in solution containing stabiliser and 0.05% sodium azide.

CD133 is a relatively novel 5-transmembrane cell surface antigen (Miraglia S 1997). The CD133/2 (clone 293C3) antibody recognises an epitope of the CD133 antigen (Yin A *et al* 1997). The epitope is called epitope 2 in order to distinguish it from another epitope which is recognised by different clones. In the haematopoietic system, CD133 expression is limited to a subset of CD34 bright stem and progenitor cells in human fetal liver, peripheral blood, cord blood and bone marrow (Buhring H *et al* 1999). In 2000, CD133 was found to be expressed on circulating endothelial progenitor cells (Gehling U *et al* 2000; Peichev M *et al* 2000) as well as on other tissue-specific stem cells. Therefore, in this case, the CD133/2 antibody (CD133/2-PE: cat #130-090-853 Miltenyi Biotec) was used to identify and enumerate CEPs and isolate them from the rest of the CD34^{+} population.

The immunofluorescent staining was carried out by taking the positively-selected CD34^{+} cell fraction produced from the previous magnetic separation step, already resuspended in 100μL of MACS buffer and divided into 2 in order to allow for a

negative control. 10μL of the CD34-FITC and 10μL of the CD133/2-PE antibodies were then added to one sample. This sample was labelled 'positive'. 10μL of negative control mouse Ab-FITC tag and negative -PE were added to the other (cat # 130-091-837 and #130-091-835 respectively). This was to act as the 'negative' control and the same process was carried out for each of the samples. Each was then mixed well and placed at $4^{\circ}C$ for 10 minutes in the dark. When working with more than 10^7 cells, reagent volumes were scaled up e.g. with 2×10^7 cells, twice the volume of all reagent and total volumes were used. The cells were then washed by adding 1-2mL of MACS buffer per 10^7 cells and spun in the centrifuge at 300g for 10 minutes. The supernatant was then aspirated completely, and the pellet resuspended in 100μL of FACS buffer prior to FACS analysis (see Appendix 1).

2.2.3 Flow Cytometry and Fluorescence activated cell sorting (FACS)

Flow cytometry has long been a widely accepted tool for immunophenotyping of cells. It offers a rapid, objective and quantitative method for analysis and purification of cells in suspension. The concept of flow cytometry is based on cells or other particles interacting with a light beam as they pass by single file in a liquid stream. A number of detectors are aimed at the point where the stream passes through the light beam: one in line with the light beam (forward scatter) and several perpendiculars to it (side scatter) (**figure 2.5**). If a fluorochrome is specifically and stoichiometrically bound to a cellular component, the fluorescence intensity will ideally represent the amount of that particular cellular component. Multiparameter flow cytometry, as used in this study, allows high-accuracy estimation of relative quantities of a variety of cells simultaneously. When measurements are recorded in a list mode, it is possible to attribute each of the several measured features to a particular cell and thus to obtain

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Figure 2.5: Forward scatter / side scatter – flow cytometry schematic

The schematic demonstrates the way in which both forward and side-scatter are used to generate data regarding cellular composition and size in flow cytometry.
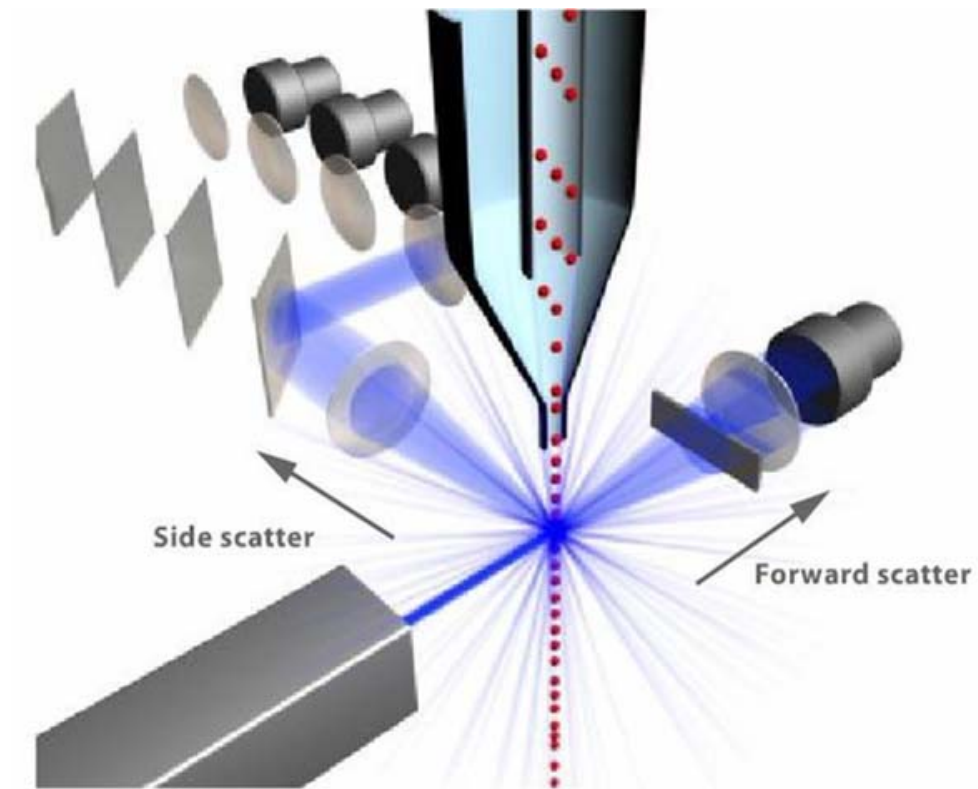
correlated measurements of these features on a cell by cell basis. Cellular heterogeneity can thus be estimated and subpopulations with distinct characteristics can be discriminated (Watson J 1992; Shapiro HM 2003).

Fluorescence-activated cell sorting (FACS) is a specialised type of flow cytometry. It allows individual cells to be physically separated from larger populations, based on a composite of parameters. Any set of criteria derived from the flow cytometric analysis can be used to activate the sorting decision for the single cell. This is based on cellular quantitative expression of molecules or their combination of predefined properties. In this study, efficiency and accuracy of cell sorting is increased by prior immunomagnetic separation.

In the identification of CECs, and their subset CEPs, a cocktail of fluorochrome-labelled monoclonal-antibodies can be used. The level of antigen expression is expressed as bright, dim or negative, allowing differentiation of these cell populations in FACS analysis. Test samples, from patients with known metastatic melanoma stored from a previous study (LREC 05/Q1105/76), were used to set the appropriate anaylsis gates. The participants of this previous study had been fully informed and had consented to use of any surplus samples in future similar studies within the department. As the test samples were passed through the laser beam, forward scatter (relating to cell size) and side-scatter (relating to cell complexity and granularity) allow the generation of plots. These are combined by the FACS software in order to generate 2-dimensional dot or scatter plots which allow differentiation of the cell populations based on fluorescence intensity. This generated series of subset extrations are termed 'gates'. A forward scatter threshold was set in all analyses for this study in order to eliminate small cells and debris. A typical blood cell run would show

distinct cell populations (using CD45-PE) i.e. lymphocytes, monocytes and neutrophils (see **figure 2.6**). Each marked or labelled cell generates a certain amount of light, depending on the fluorescence, and this is converted into a voltage pulse, which is in turn converted into a numerical value, based on voltage pulse area or height. This value is then used to plot the intensity of the event, which is produced on a log histogram where cell count is plotted on the y axis, and fluorescence on the x axis. The use of 2 different fluorochromes i.e. Ab-FITC and Ab-PE in each cell sample tested which have different emission properties allows generation of a 4 quadrant dot/scatter plot. .

Based on cell position in the forward versus side-scatter histogram, a distinct population can be gated around. This was done by drawing a box using the FACS software, around the cell population of interest, and from this distinct population, a more accurate histogram to demonstrate sub-sets within this, i.e.$CD34^+CD133^+$ versus $CD34^+CD133^-$ was generated. Compensation was made for spectral overlap of fluorescence by use of control samples prior to each study sample being passed through the flow cytometer. For each sample, after acquisition of at least 100,000 cells, analyses were considered as informative when adequate numbers of events i.e.>100, were collected in the CECs and CEPs enumeration gates. Data was then analysed using the BD FACSCalibur (BD Biosciences UK) software - CellQuest. Optimisation of the technique will be discussed in chapter 5.

2.2.4 Statistical analysis of results generated from FACS

Statistical analysis was performed using SPSS. The Kolmogorov-Smirnov test was used to determine if the CEC and CEP were normally distributed. The null hypothesis of normality was rejected if the p value of the Z statistic was < .05. The measurements

Figure 2.6: Typical blood cell run showing distinct cell populations: lymphocytes, monocytes and neutrophils.

FSC = forward scatter
SSC = side scatter

These examples are from test samples done for this study showing the three distinct cell populations and the generation of a log histogram from the scatter plot. This is using CD45-PE.
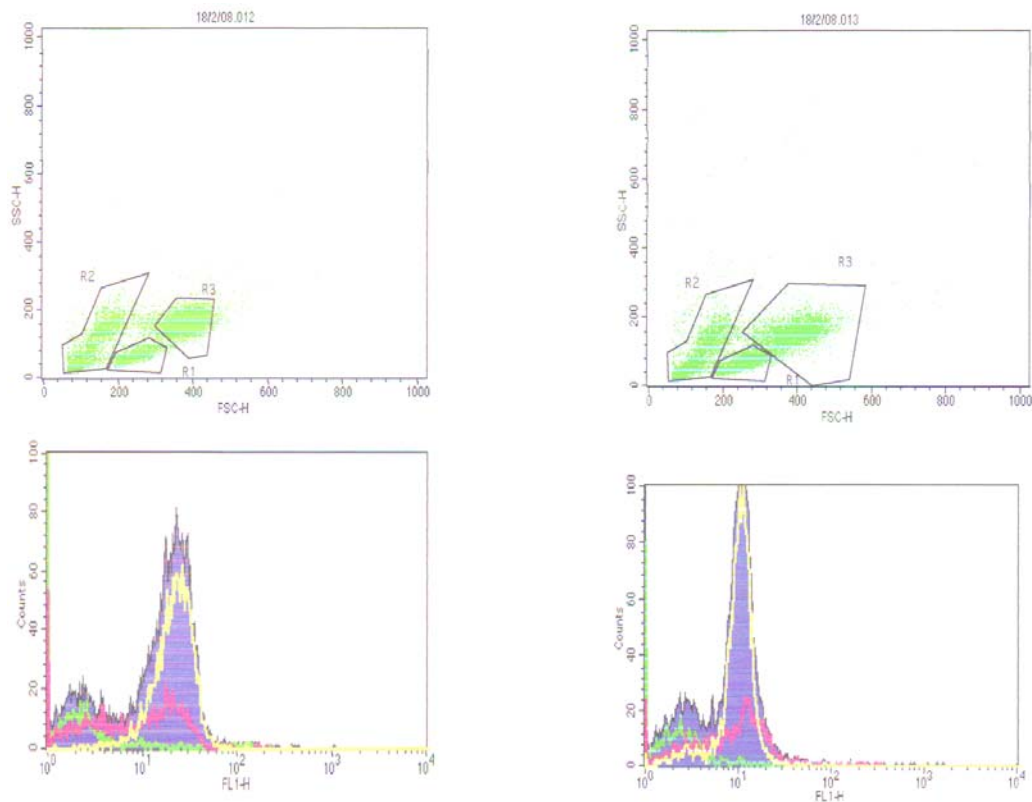
of CEC and CEP were summarized using descriptive statistics (means, standard deviations, and 95% confidence intervals). One-way analysis of variance (ANOVA) was used to test the null hypothesis that the mean baseline (pre-treatment) CEC and CEP values were the same in the three groups. One-way repeated measures ANOVA was used to test the null hypothesis that the mean differences between the repeated measures (pre-treatment minus mid-treatment, or pre-treatment minus post-treatment) were the same in the two treatment groups. The null hypothesis was rejected if the p value of the F statistic was < .05. Effect sizes were compared using the Eta^2 statistic. Levene's test was used to check for equality of variance. The null hypothesis of equality of variance was rejected if the p value of Levene's statistic was < .05. Statistics advice was taken from Fishers statistics online tool (www.fisherstat.com) and Dr S Wright – University of Cardiff.

Chapter 3

Chemotherapeutic and anti-angiogenic agents given as single agents in an extended-dosing schedule using the *in vitro* MVEC model.

3.1 Aims and Introduction

The aims of the first part of the *in vitro* study described in this chapter are:

1. To establish the *in vitro* model, using micro-vascular endothelial cells

2. To test the anti-endothelial and anti-cancer effects of oral chemotherapeutic agents and specific anti-angiogenic agents given in an extended low dosing schedule, given as single-agents

3. To determine the 'metronomic' dose of oral chemotherapeutic agents based on the dose at which significant anti-endothelial effects were seen. Suitable single-agents, at these determined doses, will be taken forward to use in combination with specific anti-angiogenics (described further in chapter 4).

The strategy of metronomic chemotherapy (see section 1.3.1 and 1.3.2), especially in combination with novel specific anti-angiogenic agents (section 1.3.4), has shown promising outcomes in pre-clinical and clinical studies (Bottini A *et al* 2006; Colleoni M *et al* 2006; Lam T *et al* 2007; Lee S *et al* 2007; Vrendenburgh J *et al* 2007; Garcia A *et al* 2008). However, there are still numerous uncertainties surrounding this strategy. These include identifying the most suitable cytotoxic drugs; establishing the optimal dosing and frequency schedule; determining the biological activity through surrogate markers and determining the potential acquirement of drug resistance (Maraveyas A *et al* 2005). All these uncertainties require as many answers as possible for each drug and potential combinations of drugs before they go forward into the

oncology clinic to be used in treatment regimens (Lam T *et al* 2007). With the proliferation of agents, an increasing number of which are now available in the oral form, there is a need to develop a useful screening tool for defining these uncertainties. Since the underlying principle of metronomic scheduling is to induce an anti-angiogenic effect, the use of an *in vitro* endothelial model provides a useful starting point from which more extensive investigations can be carried out.

Given that the term 'metronomic' seems to encompass a fairly broad dose range (10-33% of MTD – section 1.3) it is not clear whether there is a greater or smaller benefit at higher levels of this range. The clinical principle formulated for these pleiotropic cytotoxic agents (both anti-tumour and anti-angiogenic) is that one should aim to deliver as high a dose as possible within the metronomic range before conventional bone marrow toxicity is seen (Maraveyas A *et al* 2005). The avoidance of bone marrow toxicity is crucial given that bone-marrow recovery is a profoundly pro-angiogenic event and would potentially negate any anti-angiogenic gains.

The concept of 'high-time' chemotherapy, defined as the longest possible period of drug exposure at a given desired drug concentration, is very useful for long-term therapy, but requires careful assessment of the pharmacokinetics and pharmacodynamics of the drugs involved (Kamen B *et al* 2000). Dose-density chemotherapy, however, is completely different, being based on administering multiple cycles of conventional dosed cytotoxic doses of chemotherapy often with the support of growth factors, with short intervals in between weekly or fortnightly schedules, to reach the highest possible dose (Fornier M *et al* 2007). It is therefore necessary to develop tools that allow determination of the correct optimal dose for

these cytotoxics to be given in extended schedules, as opposed to an empirically devised 10-33% of traditionally calculated MTD. This form of dosing 'mantra' in the absence of distinguishable supporting scientific principles may hinder acceptance and development of these strategies.

The *in vitro* MVEC model is used in this study calls for a more rigorous determination of the 'metronomic' dose. This is based on the dose at which statistically significant *in vitro* endothelial cell inhibition occurs, rather than on an empirical value. Some inter-assay standardaization is possible this way and a comparison between the effects of different agents can be more robust. The dosing at which this is achieved can be extrapolated to known conventional dosing schedules of this drug, hence giving an indication of the potency of the dose at which 'metronomic' effects are seen in the *in vitro* model we describe. *In vitro* studies cannot be directly transposed to the clinic, but provide a starting point as to which of the many traditional agents might be suitable for the anti-angiogenic approach, and a guide-line as to which dose levels induce an anti-endothelial response.

3.1.1 Rationale of using MVEC proliferation as a surrogate marker of angiogenesis *in vitro*.

Angiogenesis involves endothelial cells in three principal events, namely migration, proliferation and maturation (Griffioen A *et al* 2000). The proliferation of endothelial cells is therefore a crucial step in the formation of tumour vasculature and many *in vitro* models of angiogenesis include this as a key parameter. A number of previous studies have looked at the antiangiogenic activity of metronomic chemotherapy. The

first *in vitro* 'metronomic' protocol was devised by Bocci and co-workers (Bocci G *et al* 2002). They looked at the effects of several cytotoxic agents including paclitaxel and cyclophosphamide on human microvascular and macrovascular endothelial cells, fibroblasts, drug-sensitive or multidrug-resistant breast cancer cell lines in cell culture. This was using either short-term (i.e. 24 hours) or long-term (i.e. 144 hours) continuous drug exposure times where the media was replaced every 24 hours. They found that continuous long-term exposure protocol demonstrated a dramatic trend of comparative vascular endothelial cell hypersensitivity in terms of growth inhibition to low drug doses compared with the other cell types.

Following on from this, further studies have been carried out whereby *in vitro* endothelial cell proliferation is used as a marker of angiogenesis (Vacca A *et al* 1999; Yap R *et al* 2005; Lam T *et al* 2007). Anti-angiogenic efficacy in these studies has been shown to be enhanced when cytotoxic drugs were administered in 'metronomic' schedules and also when combined with molecularly-targeted anti-angiogenic agents. Clinical trials have been underway over the last seven years to confirm these pre-clinical findings, showing promising results (Colleoni M *et al* 2001; Buckstein R *et al* 2006; Colleoni M *et al* 2006; Orlando L *et al* 2006).

However, the use of *in vitro* models based on MVECs or any other endothelial cell type to represent tumour endothelium is not without problems. The simplified demonstration of inhibitory effects on an endothelial cell model *in vitro* fails to take into account the anatomical complexity of tumour-related vasculature, including the stromal components. No *in vitro* model is truly representative of the *in vivo* process, and should always be regarded as an approximation as opposed to a true

representation.

In vitro chemosensitivity assays, however, do play a crucial role in the development of novel therapeutic strategies, especially when devising phase I clinical trials (Schrag D *et al* 2004). They are easily reproducible, and provide a useful guide to define dose ranges, determine potentially useful combinations and determine the presence of synergy between agents. The use of microvascular endothelial cells, as in this study, has been shown to be more closely representative of the microvasculature within tumours than HUVECs (Bocci G *et al* 2002; Lam T *et al* 2007) or other endothelial cell types. This adds weight to our findings.

3.1.2 Selection of cytotoxic drugs for metronomic chemotherapeutic dosing and the rationale behind the chosen dose-range

The following cytotoxic drugs are all traditional chemotherapeutic agents that have been used in the oncology clinic in the conventional sense i.e. MTD, for the treatment of a range of adult malignancies. The explanation and justification for using each of these agents in a metronomic schedule is given below. The dose-range chosen for each cytotoxic agent tested is also explained.

3.1.2.1 Temozolomide

Temozolomide is an alkylating agent descended from the anti-melanoma compound dacarbazine (DTIC). It is an analogue of mitozolomide, and when ingested, rapidly degrades to a highly reactive cation that methylates guanines in DNA, causing base pair mismatch. This eventually leads to inhibition of mitotic division via permanent

damage to the daughter strand (Darkes MJM *et al* 2002). It is therefore a prodrug, in that it is converted to its active product on ingestion, and has excellent bio-distribution (Newlands E *et al* 1997). It was originally developed as an alternative to DTIC – which requires metabolic activation **(see Figure 3.1a** for structure). It is now licensed for use (FDA approval) as an oral agent in the treatment of metastatic melanoma and certain brain tumours – refractory anaplastic astrocytoma (Dhodapkar M *et al* 1997; Darkes *et al* MJM 2002; Azzabi A *et al* 2005).

Since temozolomide is suitable for use in metronomic dosing schedules and also has generic anti-melanoma properties, it is an obvious compound to be included in this study (mention prodrug derivative). It is also one of the agents which comprise the treatment regimen for patients recruited into the clinical study (Chapter 5) – one group being on conventional intravenous DTIC and the other being on metronomically dosed Temozolomide.

In Phase I clinical studies of Temozolomide, daily oral 5-day schedules of the drug demonstrated a maximum tolerated dose of $250mg/m^2/day$, with thrombocytopenia and neutropenia as major dose limiting toxicities (Dhodapkar M *et al* 1997). At $50mg/m^2/day$ and $225mg/m^2/day$, after 5 days of treatment, the C^{max} values attained were 16μM and 63.9μM respectively. This provided preliminary evidence to support the use of Temozolomide in a combined metronomic protocol at 1-5% of MTD.

A further Phase I study was conducted by Brock and colleagues in patients with malignant glioma (Brock C *et al* 1998). This explored the dose-limiting toxicity and MTD of Temozolomide when administered as a single, daily dose for 6-7 weeks.

They found that grade 4 myelotoxicity was seen at 100mg/m^2/day, forcing dose reductions to 75mg/m^2/day. At this level, haematological toxicities did not exceed grade 2 in ten patients treated. Significant clinical responses were seen in this patient group, and another more recent phase I trial confirmed these results, using a 21 day out of 28 day dosing schedule. A further study by Kurzen and colleagues showed that non-toxic doses of Temozolomide inhibited endothelial cell proliferation and differentiation (Kurzen H *et al* 2003).

Temozolomide has been tested in vitro on HUVECs in combined metronomic schedules previously (Lam T *et al* 2007) and revealed promising results. On its own, Temozolomide inhibited HUVECs specifically at doses as low as 2.5µM (1-5% of MTD). In light of this, a rational approach towards defining starting doses for cytotoxic agents given in such metronomic schedules was proposed (Maraveyas A *et al* 2005). The dose range selected here (0-50µM) was therefore based on the previous *in vitro* work carried out and also by calculating the MTD from phase I data, and ensuring that the dose range selected covered a wide range but was well below the empirically recommended upper limit of 33% of MTD (see **table 2.1** for summary).

3.1.2.2 Paclitaxel

Paclitaxel is a mitotic inhibitor that acts at a microtubular level. It was first isolated from the bark of the Pacific yew tree, Taxus brevifolia, four decades ago. This again is a well tolerated orally bioavailable agent, and has shown promising anti-tumour activity in a number of solid tumours including ovarian, breast, lung and prostate. See **Figure 3.1b** for chemical structure. It has proven efficacy in metronomic regimens, both as a single agent and in combinations in both *in vitro* and *in vivo* studies.(Belotti

D *et al* 1996; Bocci G *et al* 2002; Grant DS *et al* 2003; Wang J *et al* 2003; Lam T *et al* 2007). Anti-angiogenic properties have been shown by paclitaxel's ability to inhibit endothelial cell proliferation at concentrations lower than those required to inhibit tumour cell proliferation (Belotti D *et al* 1996; Drevs J *et al* 2004; Albertsson P *et al* 2006).

Previous pharmacokinetic studies involving paclitaxel have shown that 6 hour infusions and 24 hour infusions, each administered once every 3 weeks, resulted in an MTD of 250mg/m^2 and corresponding C_{max} values of 13µM and 1µM respectively (Rowinsky EK *et al* 1992). The data suggests that paclitaxel may be used at less than 15% MTD as part of a combined 'metronomic' protocol to induce a significant anti-endothelial response. Bocci and colleagues demonstrated that ECS scheduling with several cytotoxic agents including paclitaxel caused significant apopotosis in endothelial cells after 6 days of treatment (Bocci G *et al* 2002). This was also demonstrated by Wang and colleagues (Wang W and Passaniti A 1999; Grant DS *et al* 2003). Other potential anti-angiogenic mechanistic effects of paclitaxel that have been demonstrated include reduction in endothelial cell interstitial pressure (Griffon-Etienne G *et al*1999), increases in matrix metalloproteinases (MMP) and increases in tissue inhibitor of matrix metalloproteinases (TIMP) (Alonso DF *et al* 1999). Mention Phase I pharmacokinetic data (see table 2.1)

Paclitaxel is currently used mainly in its intravenous form. It has a low therapeutic index and is virtually insoluble in water, hence its mixture with Cremophor EL – a mixture of ethanol and polyoxyethylated castor oil. Initial trials into its use as an oral compound showed poor bioavailability, most likely due to its affinity to the membrane-bound drug efflux pump P-glycoprotein (P-gp). However, when

administered with P-gp inhibitors, such as cyclosporin-A, oral bioavailability was greatly enhanced. This has shown potential in Phase II studies, making paclitaxel a potentially significant metronomic agent of the future (Yang S *et al* 2004). Paclitaxel was therefore deemed a suitable agent to use in this study, and interesting to compare the effects on MVECs to the effects on HUVECs as previously studied in our group (Lam T *et al* 2007). The dose range selected (0-2nM) was therefore based on the previous *in vitro* work afore mentioned and phase I data, covering a wide range of doses, all less than 20% of the MTD.

3.1.2.3 Vinorelbine

This is the first semi-synthetic vinca alkaloid, obtained from alkaloid extracts obtained from the rosy periwinkle (see **Figure 3.1c** for chemical structure). It gained approval to treat non-small cell lung cancer in 1991, and has since been used in the treatment of breast and prostate cancer also (Rowinsky EK *et al* 1994). An oral formulation was licensed for use in 2004 which has similar efficacy and safety profile to the intravenous form. This makes it another agent potentially suited to the metronomic approach and for use in this study (Rowinsky EK *et al* 1994; Marty M *et al* 2001). It has similarly been studied using an *in vitro* human endothelial cell model to assess its antiangiogenic effects, although clinical extrapolation of these princliples are still awaited (Pappas P *et al* 2008).   Vinblastine, a family member of vinorelbine, has also been shown to have impact on *in vitro* endothelial cell function, proliferation, chemotaxis and vascular morphogenesis (Vacca A *et al* 1999).

Phase I pharmacokinetic data showed that after oral administration of 80mg/m^2, vinorelbine was rapidly absorbed with a mean peak blood concentration (C$_{max}$) of

133.4 ± 42.3 ng ml^{-1} at 1.4 ± 0.7 hr (T$_{max}$). The dose range selected for vinorelbine in this study was therefore based on phase I data and on previous *in vitro* studies. 0-33% of MTD correlated to a dose range of 0-10 µM (see **table 2.1**).

3.1.2.4 Etoposide

Etoposide phosphate is an inhibitor of the enzyme topoisomerase II, with anti-mitotic and antineoplastic properties (see **Figure 3.1d** for chemical structure). It is used in the treatment of Ewing's sarcoma, lung cancer, testicular cancer, leukaemias and lymphomas. It can be given in the oral or intravenous form, with comparable efficacy, again making it an appropriate agent for the anti-angiogenic approach (Markman M *et al* 1992; Sessa C *et al* 1995). Phase II trials have shown that, given in an extended dosing schedules, results are comparible with conventional dosing regimens in lung cancer, although further studies are required (Grunberg S *et al* 1993; Greco A and Hainsworth J 1994). The clinical formulation does however suffer from variable absorption and unpredictable bone marrow toxicity (Sessa C *et al* 1995).

It has been shown to induce endothelial cell inhibition, with IC$_{50}$ values significantly lower than tumour cells in previous *in vitro* studies using the HUVEC model, but this has not been demonstrated on an MVEC model before (Drevs J *et al* 2004). The dose range selected for etoposide in this study was therefore based on phase I pharmacokinetic data from intravenous administration. Sessa and colleagues determined that MTDs were defined as 175mg/m^2 and 220mg/m^2 in previously treated and untreated patients respectively (Sessa C *et al* 1995). 0-33% of the calculated MTD translated to a range of 0 to 5µM, the dose range used in this study (see **table 2.1**).

3.1.2.5 Carboplatin

Carboplatin is a chemotherapy drug mainly used against ovarian carcinoma, lung and head and neck cancers. It was introduced in the late 1980s and has since gained popularity in clinical treatment due to its vastly reduced side-effects compared to its parent compound cisplatin (see **Figure 3.1e** for chemical structure). Cisplatin and carboplatin as well as oxaliplatin, are classified as DNA alkylating agents. Carboplatin causes highly reactive platinum complexes to form intracellularly and therefore inhibits DNA synthesis.

Phase I data suggested that the MTD of carboplatin was upto 1000mg/m^2 (Gore M *et al* 1987; Shea T *et al* 1989; Yoshizawa H *et al* 2003), and it is currently administered conventionally in doses of 600-700mg every 3-4 weeks. It is almost exclusively excreted by the kidneys, and therefore the total body clearances of ultrafiltratable platinum and that of the parent carboplatin molecule correlate linearly with the pre-treatment glomerular filtration rate. Because of this, physiologic variables such as renal function, can affect the plasma concentration of carboplatin in proportion to total body clearance. Calvert and colleagues devised a formula which would allow for a degree of compensation for physiologic variables, taking into account the renal clearance (Calvert AH *et al* 1989). This minimised resultant toxicities (Alberts D and Dorr R 1998; Sato E *et al* 2006).

Carboplatin as an oral agent, is currently being studied, and is again an interesting compound to include in this study. The phase I data (Gore M *et al* 1987) allowed correlation to a suitable dose range for this study, and was calculated as 0-500μM.

Figure 3.1: Chemical Structures of Cytotoxic Agents

a)Temozolomide
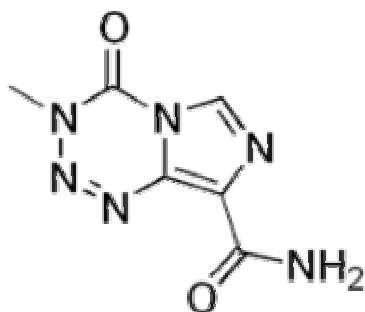
b) Paclitaxel
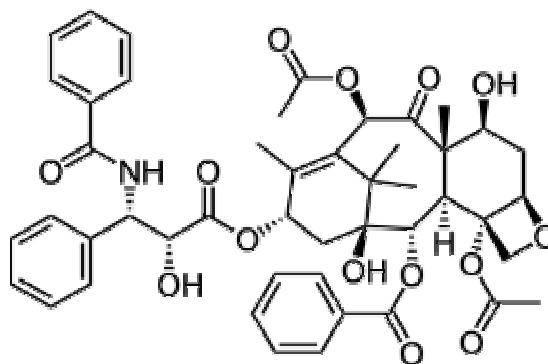
c) Vinorelbine
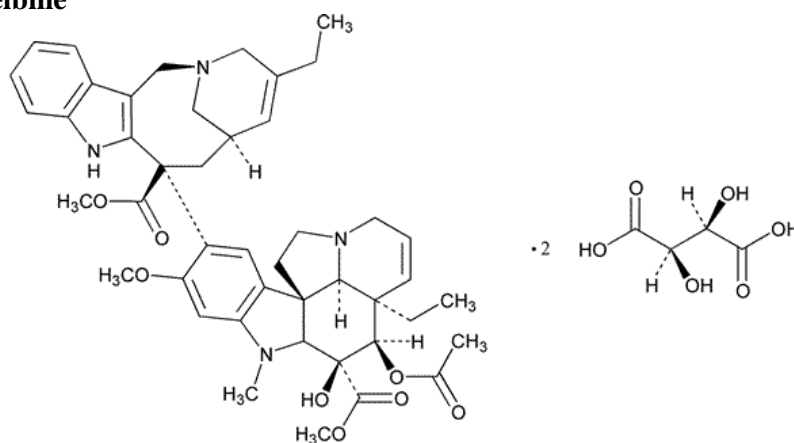
d) Etoposide
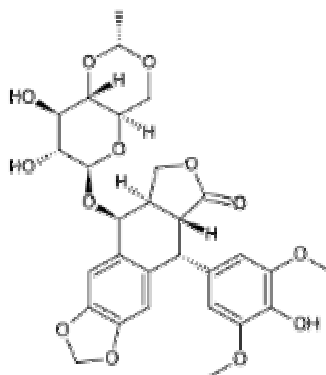
e) Carboplatin
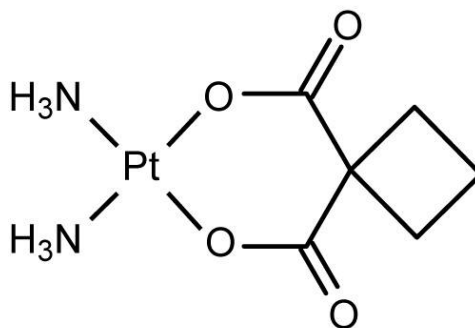
3.1.3 Selection of drugs for specific anti-angiogenic agents

It is very important to make a clear distinction here between specific anti-angiogenic agents and cytotoxic agents being administered for an anti-angiogenic effect. A metronomic dose is not required for specific agents, as the pre-determined dose for these agents is already devised based on a safe, daily, oral regimen and they have been devised specifically to induce a targeted effect. The cytotoxics, however, have up until recently, only ever had MTDs attributed to them. A dose at which anti-endothelial effects are seen has therefore yet to be determined, which is the underlying basis of this study.

3.1.3.1 Sorafenib

As previously stated (section 1.2.5), this is a small molecule inhibitor of the raf kinase, platelet-derived growth factor, VEGF receptors 2 and 3 kinases, and the c kit receptor (see **Figure 3.2a** for molecular structure). It prevents tumour growth by combining its anti-angiogenic and anti-proliferative effects (Wilhelm S *et al* 2004). Anti-proliferative activity has been observed in tumours with Ras mutations, as well as those in which Ras is activated through activation of growth factor receptors and with B-Raf mutations. It has so far shown varying success in clinical trials of renal cell carcinoma, hepatocellular carcinoma, malignant melanoma and lung cancer (Hanahan D and Folkman J 1996; Mross K *et al* 2006; Llovet J *et al* 2008). This drug is administered as on oral agent at a dose of 200-400mg twice a day. This translates to a suitable AUC of 10 µM. Drug concentrations reflecting this dose were used for the combination experiments.

3.1.3.2 Combretastatin (CA4P)

This is a vascular targeting and anti-angiogenic agent, originally isolated from the southern African shrub, Combretum caffrum (see **Figure 3.2b**) for molecular structure). It has been shown to specifically target proliferating endothelial cells and to block mitosis through binding to tubulin causing destabilisation of the cytoskeleton and thus inducing apoptosis (Dorr R *et al* 1996; Dark G *et al* 1997). This leads to blood flow shut down to tumours rather than to surrounding normal tissues ultimately producing tumour cell death through oxygen and nutrient starvation. It therefore causes tumour necrosis, whilst also displaying antiangiogenic properties, being toxic to actively proliferating vascular endothelial cells and various cancer cells at low micromolar concentrations (Dorr R *et al* 1996; Vincent L *et al* 2005). In contrast to other tubulin-binding agents that have significant dose limiting toxicity, CA4P is active at one tenth of its MTD giving it an extremely wide therapeutic window (Dark G *et al* 1997). CA4P has shown promising results in clinical trials as a single agent and at present is entering a range of combinational therapy trials including a phase I trial with bevacizumab and a phase II trial with doxorubicin and cisplatin in patients with newly diagnosed thyroid cancer (Cooney M *et al* 2005; Nathan P *et al* 2008). It is also an orally available agent (Bilenker J *et al* 2005).

We preferred to classify this agent as a non-specific antiangiogenic agent more akin to chemotherapy rather that a specific agent despite the main body of literature alluding to it as an anti-angiogenic molecule. From phase I pharmacokinetic data, the dose range selected for CA4P was therefore 0-25nM within which the 'metronomic' range 10-33% would have been encompassed. .

3.1.3.3 Bortezomib

Bortezomib is the first therapeutic proteasome inhibitor to be tested on humans, and is now approved in for treating relapsed multiple myeloma and mantle cell lymphoma in the US (Druck M *et al* 2006). FDA approval was granted for this based on striking phase II data (Richardson P *et al* 2003). It is a low-molecular-weight dipeptide which inhibits the proteosome by binding to chymotryptic-like enzymes (see **Figure 3.2c** for molecular structure). This prevents degradation of pro-apoptotic factors, permitting activation of apoptosis in neoplastic cells. It is therefore not a specific anti-angiogenic agent, although it is a molecularly targeted agent, which has good oral bioavailabilty. It antiangiogenic properties have been investigated in both preclinical and clinical studies, showing some efficacy – contributing to its success in treatment of multiple myeloma (Politou M *et al* 2005). This makes it suitable for comparative purposes in this study.

Phase I data suggested MTD to be $1.3mg/m^2$ (Dees E *et al* 2008). A dose range including this dose within which the 'metronomic range 10-33% would have been encompassed was 0-100nM.

Figure 3.2: Chemical Structures of Anti-angiogenic Agents

a)Sorafenib
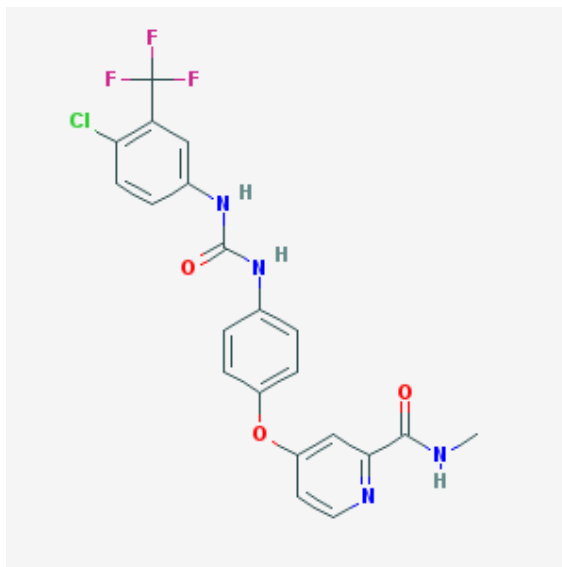
b)Combretastatin
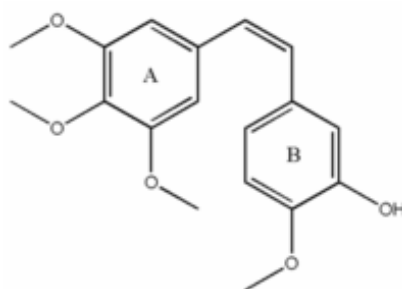
c) Bortezomib
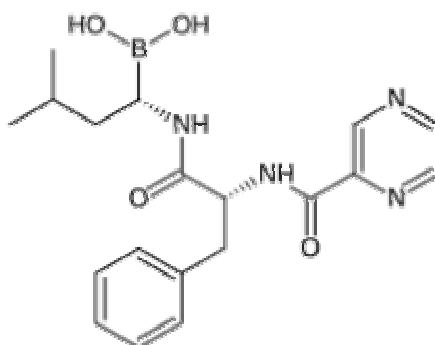
3.2 Results

3.2.1. Determination of the appropriate cell density to achieve the longest possible duration of cell growth in chemosensitivity assays

The optimal cell seeding densities were determined in order to achieve the longest period of uninterrupted growth in 96-well plates up to confluence. This was determined by MVECs, which grew for 4 days, and therefore determined the duration of drug exposure for all cells (96 hours) – see section 2.1.1.5.The longest growth period, which determined the duration of drug exposure for all cells was 4 days (96 hours). This growth period was seen in the MVECs. Optimal seeding densities were thus determined as 3500 cells/well for MVECs, and as 2000 cells/well, 1500 cells/well and 1000 cells/well for SK-MEL-2, MCF7 and A549 cell lines respectively. The calculated number of cells required in each well for each cell type remained constant for each independent chemosensitivity assay subsequently carried out for all agents tested.

3.2.2 Determination of the effects of low dose DMSO on the growth of cells and of the baseline control growth rate

The effect of different concentrations of DMSO on each cell type was determined, since the majority of the drugs tested required reconstitution in this. It was shown that there was no inhibitory or anti-proliferative effect of DMSO at concentrations less than 0.25% on all four cell lines (see **Figure 3.3**). All drugs used were therefore reconstituted in 100% DMSO and stored in 10mM solutions. From this stock solution, the aliquots of each drug concentration required were made up at 1000 times the concentrations actually required. These aliquots were diluted by a factor of 1000

Figure 3.3: Growth response of MVEC in culture media containing DMSO
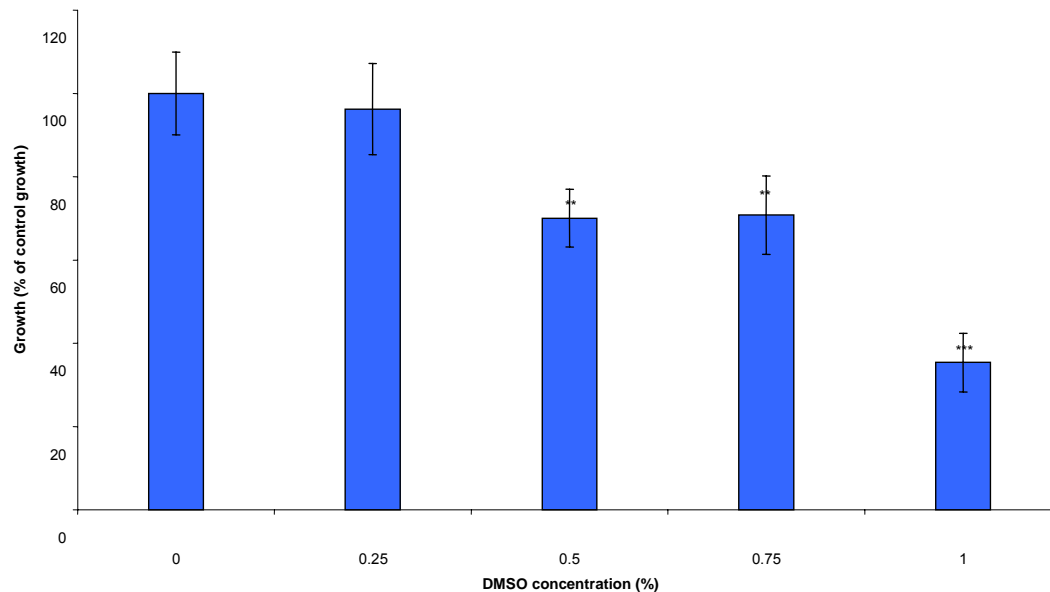
Growth of MVEC following 4 days of incubation with various concentrations of DMSO in growth media as determined by MTT proliferation assay. The purpose of the experiment was to verify that DMSO did not interfere with growth of cells, metabolism of MTT nor the final optical density reading. All absorbance readings were performed using a spectrophotometric plate reader at 492nM and individual readings were background-subtracted. The growth of untreated cells was taken as 100% and the growth of DMSO-exposed populations of cells was expressed as a % of this. The histogram bars represent mean growth values +/- standard error and statistical significance of results is shown. The data shown are representative of at least 2 independent experiments, each conducted in triplicate.

when used for the daily media+agent change, thus ensuring both correct concentration of the drug, and a constant DMSO concentration of 0.1% for all cells treated.

0.1% DMSO in medium therefore acted as control in all experiments, and allowed for the baseline control growth rate to be determined. This therefore represents untreated cells. After 4 days, the growth rates for MVECs, MCF7, A549 and SK-MEL-2 cells were 101.02% (±2.4), 98.36% (±2.89), 108.02% (±3.3) and 105.27% (±1.55) respectively. As per Johns study carried out in 2003, the percentage growth of the untreated cells after 4 days was normalized to 100%, and the growth of treated cells was there after expressed as a percentage of this baseline growth (Johns TG 2003).

3.2.3 Determination of the effects of single anti-angiogenic agents on MVECs and cancer cell lines

3.2.3.1 Sorafenib

Figure 3.4 (summary in table 3.1) shows the anti-proliferative activity of sorafenib in the aforementioned cell lines. The lowest assayed dose of sorafenib which inhibited the growth of endothelial (MVEC) cells by over 25% compared to control cell growth (70.18% of control growth ±2.39), at a highly significant p-value (p<0.0001), was 5μM.. IC_{50} was calculated at 7000nM. SK-MEL-2 and MCF7 cell lines showed significant inhibition of proliferation at concentrations as low as 0.25μM (P < 0.001) with IC_{50} values of 750nM and 3000nM respectively. A549 cell line showed significant inhibition to 28.99% of control growth (±1.52, P<0.001) at concentrations of 5μM and above.

Figure 3.4: Growth response of cell lines: Sorafenib
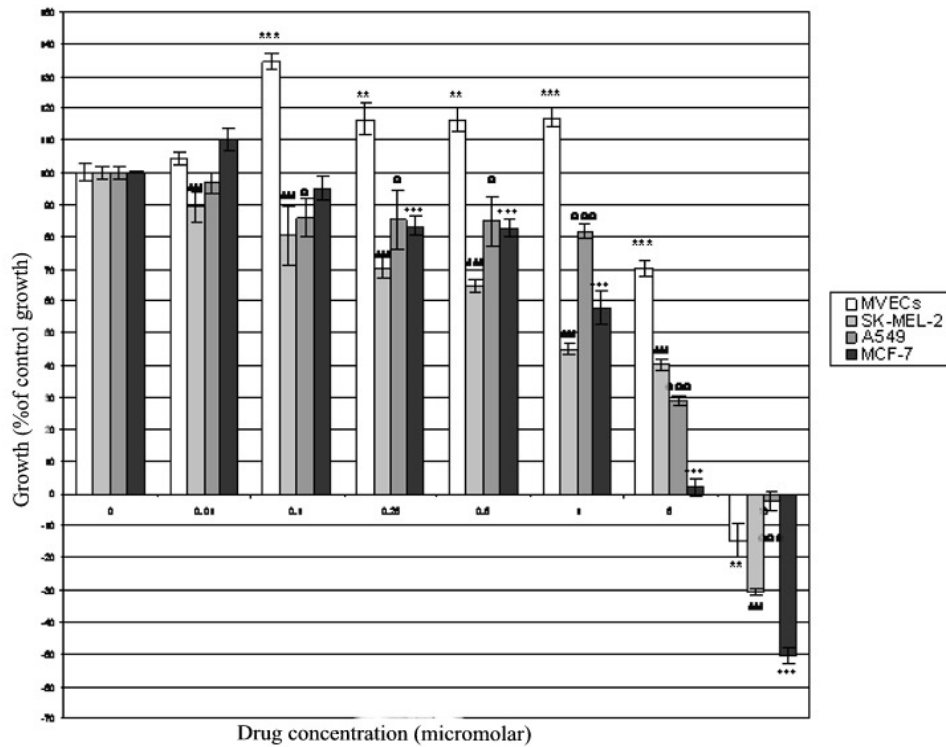
Growth of cell lines following 4 days of treatment with Sorafenib was determined by an MTT proliferation assay. All absorbance readings were performed using a spectrophotometric plate reader at 492nM and individual readings were background-subtracted. Readings at 4 hours post MTT addition were used as final readings for growth calculation. The growth of untreated cells was taken as 100% and the growth of treated populations of cells was expressed as a % of this. The histogram bars represent mean growth values +/- standard error and statistical significance of results is shown. The data shown are representative of at least 2 independent experiments, each conducted in triplicate

3.2.3.2 Combretastatin

This caused significant MVEC inhibition (**see Figure 3.5 and table 3.1**) at concentrations of 2.5nM ($p<0.01$) and above, with a calculated IC_{50} of 4.2nM. The SK-MEL-2 cell line was not affected within the selected dose range thus IC_{50} was not achieved. Both the A549 and MCF-7 cell lines were only significantly inhibited at 1 nM ($p<0.05$) and above, and an IC_{50} value was not attained for either cell line.

3.2.3.3 Bortezomib

This caused significant MVEC inhibition at concentrations as low as 1.56nM ($p<0.01$) and above, with a calculated IC_{50} of 12.2nM. The SK-MEL-2 cell line was dramatically inhibited by Bortezomib, at concentrations of 6.25nM ($p<0.01$) and above, reaching IC_{50} at a concentration of 4.7nM. A549 cell line had a similar IC_{50} value of 5.3nM, and the MCF7 cell line reached their IC_{50} value at a concentration of 16.8nM (**see Figure 3.6 and table 3.1**).

Figure 3.5: Growth response of cell lines: Combretastatin
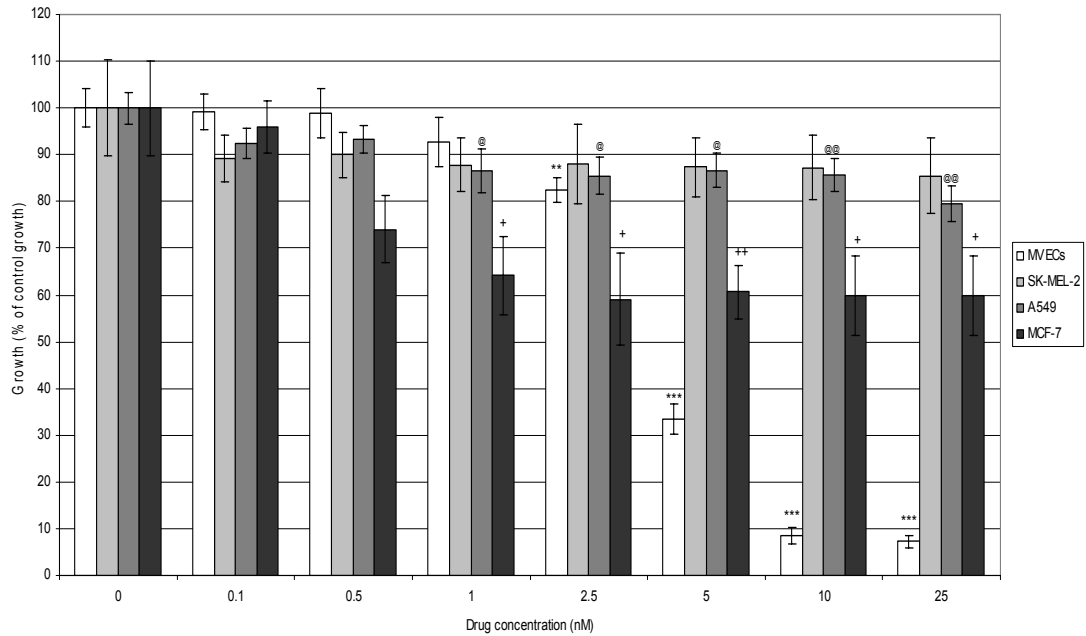
Growth of cell lines following 4 days of treatment with Combretastatin was determined by an MTT proliferation assay. All absorbance readings were performed using a spectrophotometric plate reader at 492nM and individual readings were background-subtracted. Readings at 4 hours post MTT addition were used as final readings for growth calculation. The growth of untreated cells was taken as 100% and the growth of treated populations of cells was expressed as a % of this. The histogram bars represent mean growth values +/- standard error and statistical significance of results is shown. The data shown are representative of at least 2 independent experiments, each conducted in triplicate.

Figure 3.6: Growth response of cell lines: Bortezomib
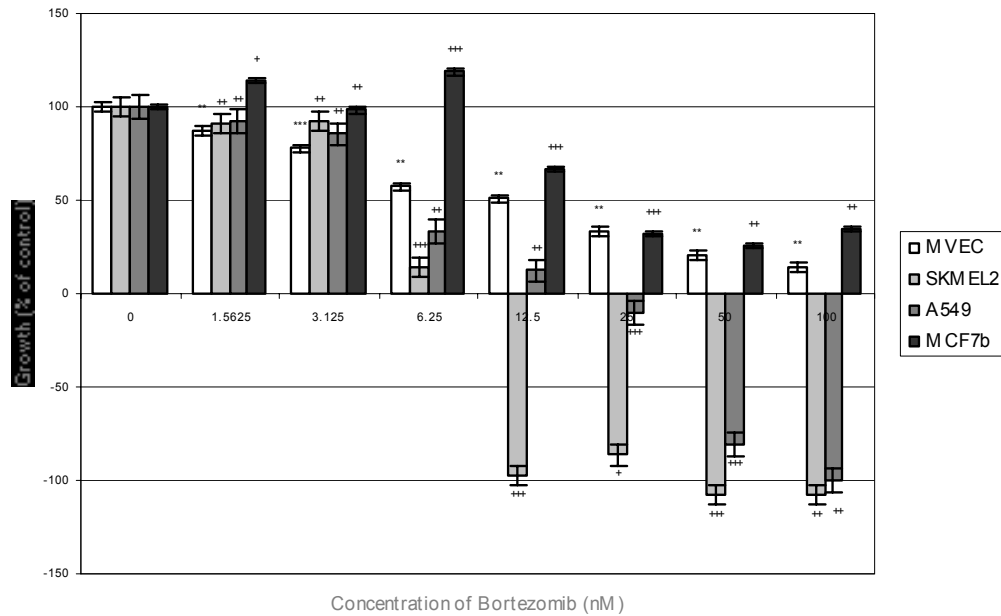
Growth of cell lines following 4 days of treatment with Bortezomib was determined by an MTT proliferation assay. All absorbance readings were performed using a spectrophotometric plate reader at 492nM and individual readings were background-subtracted. Readings at 4 hours post MTT addition were used as final readings for growth calculation. The growth of untreated cells was taken as 100% and the growth of treated populations of cells was expressed as a % of this. The histogram bars represent mean growth values +/- standard error and statistical significance of results is shown. The data shown are representative of at least 2 independent experiments, each conducted in triplicate.

3.2.4 Determination of the effects of single cytotoxic drugs given in a metronomic schedule on MVECs and cancer cell lines and determination of the metronomic dose

3.2.4.1 Temozolomide

MVECs showed anti-proliferative effects to Temozolomide in a dose-dependent manner (**Figure 3.7 and table 3.1**) with significant effects (80.89% of control growth ±3.19) seen at a concentration of 10µM and above (P < 0.001). A549, MCF7 and SK-MEL-2 cell lines all behaved in a dose-dependent manner, none achieving their IC_{50} value in the selected dose range. However, both SK-MEL-2 and MCF7 cell lines showed a significant response at concentrations as low as 1.25mM to 74.9% ± 1.24 and 76.82%± 1.53 of control growth respectively (P<0.001).

Given the literature, and the potential cumulative affect of daily dosing when shifting from MTD approach, it was decided that the 'metronomic' dose of temozolomide would be taken as 10µM. This is the dose at which statistically significant endothelial cell inhibition occurred (p<0.001), and corresponded with less than 15% of the MTD.

3.2.4.2 Paclitaxel

MVECs showed anti-proliferative effects to Paclitaxel again in a dose-dependent manner (**Figure 3.8 and table 3.1**) with significant effects (p<0.001) seen at a concentration of 125 pM. IC_{50} was achieved at 1.25nM. Both A549 and MCF7 cell lines behaved in a similar dose-dependent fashion to the MVECs achieving IC_{50} values of 1.45nM and 1.08nM respectively. The SK-MEL-2 cell line showed increased sensitivity to paclitaxel than all other three cell lines, with anti-proliferative effects observed at

Figure 3.7: Growth response of cell lines: Temozolomide
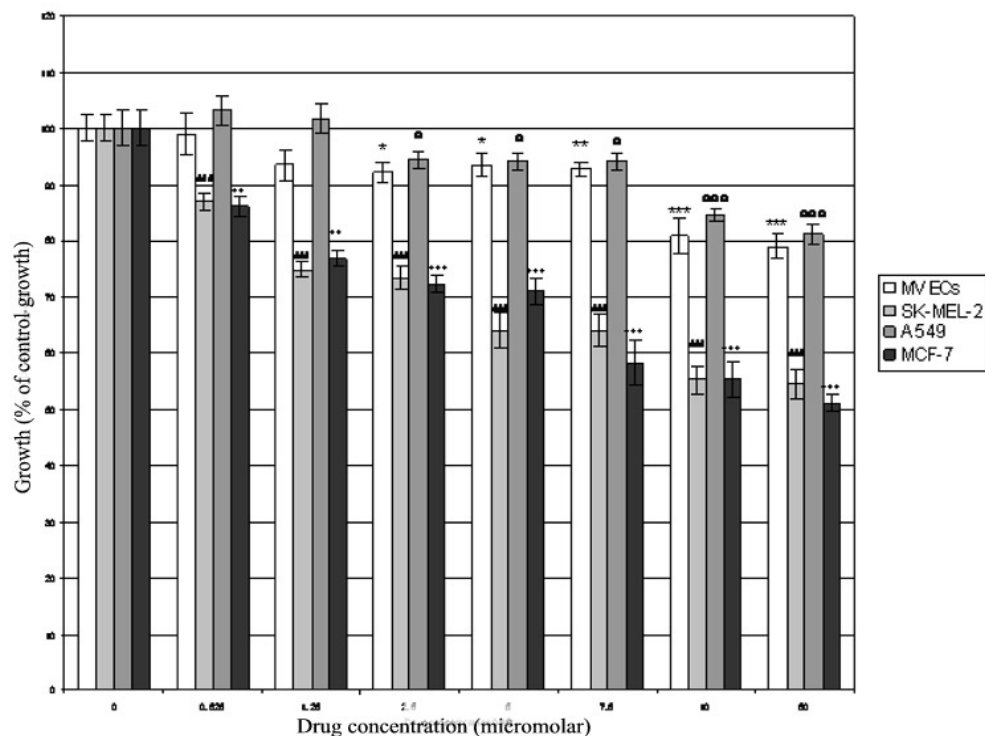
Growth of cell lines following 4 days of treatment with Temozolomide was determined by an MTT proliferation assay. All absorbance readings were performed using a spectrophotometric plate reader at 492nM and individual readings were background-subtracted. Readings at 4 hours post MTT addition were used as final readings for growth calculation. The growth of untreated cells was taken as 100% and the growth of treated populations of cells was expressed as a % of this. The histogram bars represent mean growth values +/- standard error and statistical significance of results is shown. The data shown are representative of at least 2 independent experiments, each conducted in triplicate.

Figure 3.8: Growth response of cell lines : Paclitaxel
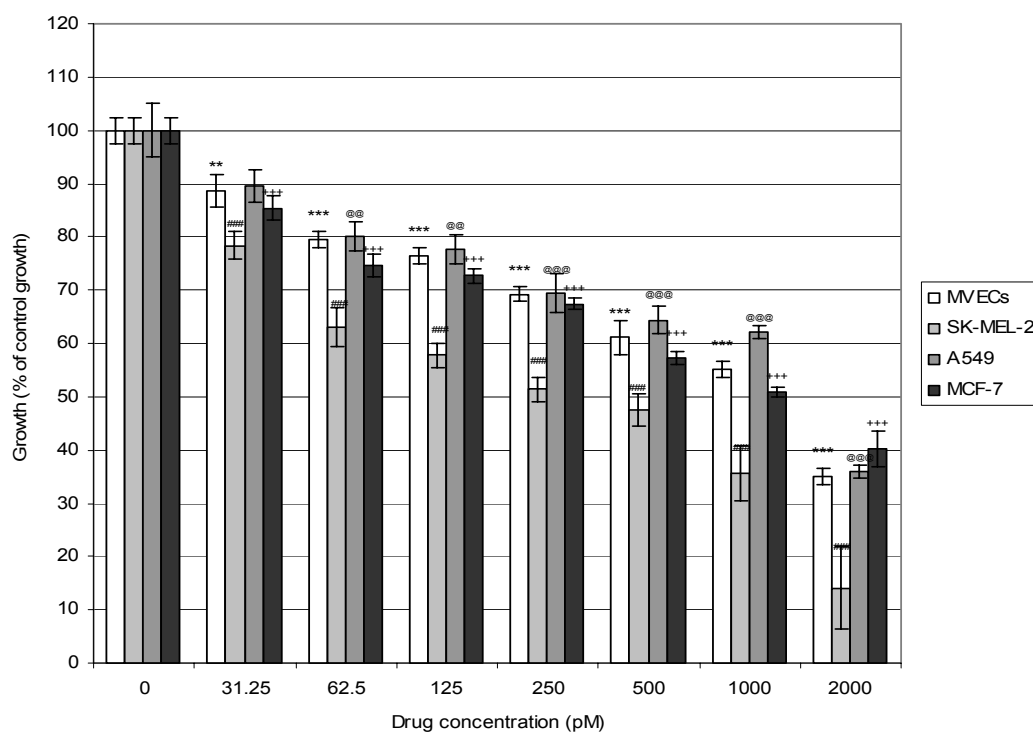
Growth of cell lines following 4 days of treatment with Paclitaxel was determined by an MTT proliferation assay. All absorbance readings were performed using a spectrophotometric plate reader at 492nM and individual readings were background-subtracted. Readings at 4 hours post MTT addition were used as final readings for growth calculation. The growth of untreated cells was taken as 100% and the growth of treated populations of cells was expressed as a % of this. The histogram bars represent mean growth values +/- standard error and statistical significance of results is shown. The data shown are representative of at least 2 independent experiments, each conducted in triplicate.

31.3pM ($p<0.001$) and above, and reaching a calculated IC_{50} at the low concentration of 350pM. The MTD of paclitaxel equates to 0.33μM, and a metronomic dose of 125pM (0.03% of MTD) was selcted based on the MTT data for MVECs.

3.2.4.3 Vinorelbine

MVECs showed significant anti-proliferative effects to Vinorelbine at doses as low as 1nM ($p<0.001$) and above, with a calculated IC_{50} value of 16.9nM (**Figure 3.9 and table 3.1**). The SK-MEL-2 and MCF-7 cell lines showed similar sensitivity to Vinorelbine as MVECs and showed significant inhibition at 1nM ($p<0.001$ and $p<0.01$ repectively) and above. A549 cell line was relatively more resistant at the low dose of 1nM (96.55%±1.26) but displayed significant anti-proliferative effects at 10nM ($p<0.01$), achieving an IC_{50} value of 55nM . All cell lines showed less than 10% of control growth at concentrations of 100nM and above, and therefore all reached IC_{50} value within the dose range selected. The MTD of vinorelbine equates to 0.123μM and a dose of 1 nM (0.8% of MTD) was selected as the metronomic dose based on the MVEC MTT data.

3.2.4.4 Etoposide

All cell lines responded to etoposide in a dose-dependent manner (**Figure 3.10 & table 3.1**). MVECs showed significant anti-proliferative effects at concentrations of 0.16μM ($p<0.001$) and above, with a calculated IC_{50} value of 1.4μM. The SK-MEL-2 cell line displayed increased sensitivity to etoposide than the other cell lines, with anti-proliferative effects observed at 0.078μM ($p<0.05$) and a calculated IC_{50} of 0.31μM. A549 and MCF-7 cell lines showed significant anti-proliferative effects at 0.16μM ($p<0.05$) giving calculated IC_{50} values of 0.58μM and 2.6μM, respectively.

Figure 3.9: Growth response of cell lines: Vinorelbine
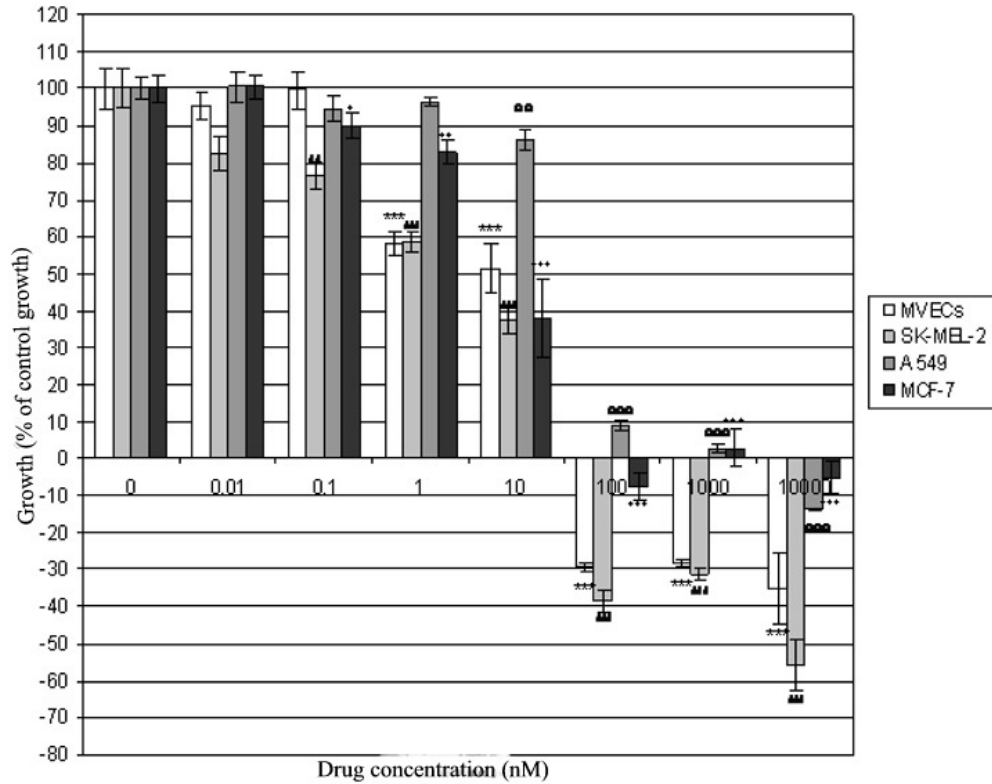
Growth of cell lines following 4 days of treatment with Vinorelbine was determined by an MTT proliferation assay. All absorbance readings were performed using a spectrophotometric plate reader at 492nM and individual readings were background-subtracted. Readings at 4 hours post MTT addition were used as final readings for growth calculation. The growth of untreated cells was taken as 100% and the growth of treated populations of cells was expressed as a % of this. The histogram bars represent mean growth values +/- standard error and statistical significance of results is shown. The data shown are representative of at least 2 independent experiments, each conducted in triplicate.

Figure 3.10: Growth response of cell lines: Etoposide
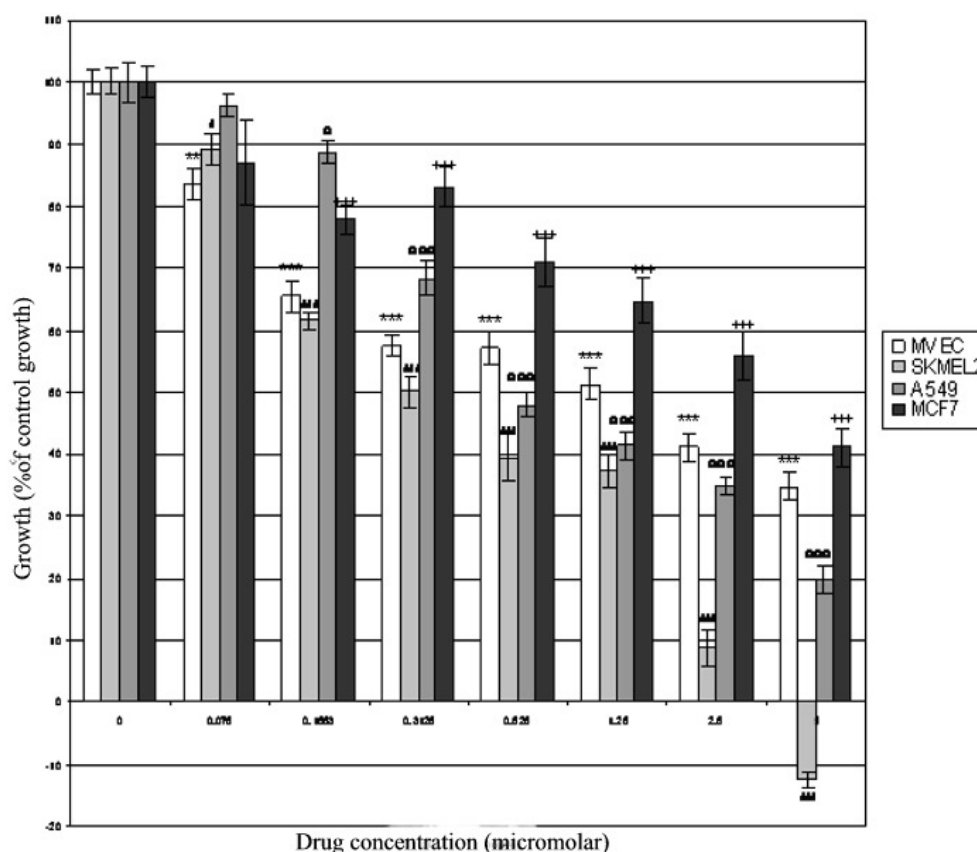
Growth of cell lines following 4 days of treatment with Etoposide was determined by an MTT proliferation assay. All absorbance readings were performed using a spectrophotometric plate reader at 492nM and individual readings were background-subtracted. Readings at 4 hours post MTT addition were used as final readings for growth calculation. The growth of untreated cells was taken as 100% and the growth of treated populations of cells was expressed as a % of this. The histogram bars represent mean growth values +/- standard error and statistical significance of results is shown. The data shown are representative of at least 2 independent experiments, each conducted in triplicate.

The MTD of etoposide equates to 35.8μM and a dose of 0.16μM (0.4% of MTD) was selected, based on the MVEC MTT data i.e. that which induced a significant anti-endothelial response (p<0.001).

3.2.4.5 Carboplatin

MVECs showed inhibitory effects to Carboplatin at doses of 250μM (p<0.01) and above, and did not reach its IC_{50} value in the dose range selected i.e. less than 33% of MTD. SK-MEL-2 and MCF-7 cell lines displayed increased sensitivity to carboplatin, with significant anti-proliferative effects seen at 0.1μM (p<0.01) for both cells lines, and reaching their IC_{50} values at concentrations of 20μM and 35μM respectively. A549 cell line gave an IC_{50} value of 72μM, and showed significant anti-proliferative effects at 50μM (p<0.01). Since MVECs did not reach their IC_{50} value in the dose range selected, a metronomic dose could not be determined for carboplatin, and it was therefore not taken forward for use in combination dosing schedules (see**Figure 3.11 & summary table 3.1**).

Figure 3.11: Growth response of cell lines: Carboplatin
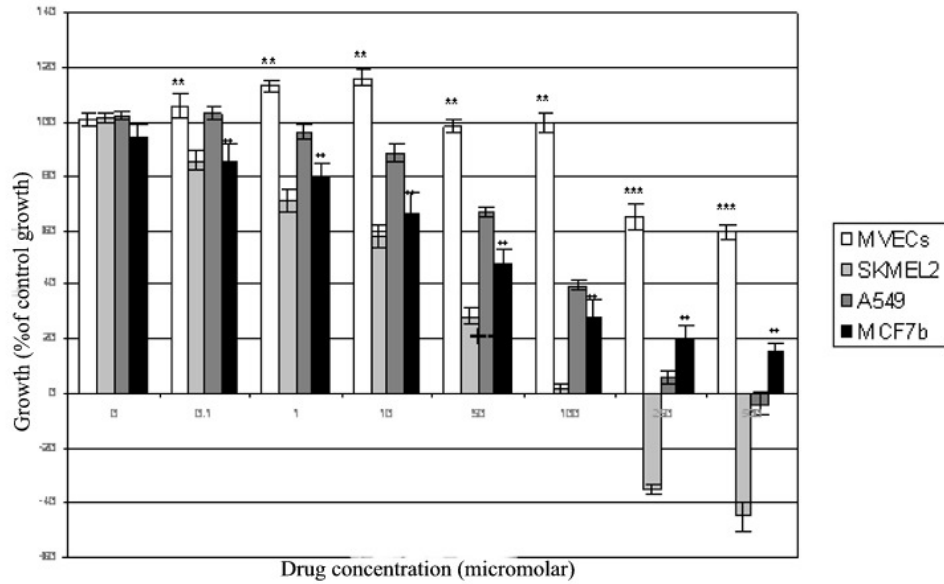
Growth of cell lines following 4 days of treatment with Carboplatin was determined by an MTT proliferation assay. All absorbance readings were performed using a spectrophotometric plate reader at 492nM and individual readings were background-subtracted. Readings at 4 hours post MTT addition were used as final readings for growth calculation. The growth of untreated cells was taken as 100% and the growth of treated populations of cells was expressed as a % of this. The histogram bars represent mean growth values +/- standard error and statistical significance of results is shown. The data shown are representative of at least 2 independent experiments, each conducted in triplicate.

Table 3.1 – Summary table of in vitro single agent data using anti-angiogenic and cytotoxic agents.

Agent + dose range used	Cell Line	Dose at which significant growth inhibition seen	P value of significant growth inhibition	IC$_{50}$ (if reached) within dose range
Sorafenib (0-10μM)	MVEC	5μM	P<0.001	7000nM
	SKMEL2	0.25μM	P<0.001	750nM
	MCF7	0.25μM	P<0.001	3000nM
	A549	5μM	P<0.001	7000nM
CA4P (0-25nM)	MVEC	2.5nM	P<0.01	4.2nM
	SKMEL2	/	/	Not reached
	MCF7	1nM	P<0.05	Not reached
	A549	1nM	P<0.05	Not reached
Bortezomib (0-100nM)	MVEC	1.56nM	P<0.01	12.2nM
	SKMEL2	6.25nM	P<0.01	4.7nM
	MCF7	12.5nM	P<0.01	16.8nM
	A549	6.25nM	P<0.01	5.3nM
Temozolomide (0-50μM)	MVEC	10μM	P<0.001	Not reached
	SKMEL2	1.25mM	P<0.001	Not reached
	MCF7	1.25mM	P<0.001	Not reached
	A549	10mM	P<0.01	Not reached
Paclitaxel (0-20nM)	MVEC	125pM	P<0.001	1.25nM
	SKMEL2	31.3pM	P<0.001	350pM
	MCF7	62.5pM	P<0.01	1.08nM
	A549	62.5pM	P<0.01	1.45nM
Vinorelbine (0-10μM)	MVEC	1nM	P<0.001	16.9nM
	SKMEL2	1nM	P<0.001	12.5nM
	MCF7	1nM	P<0.01	12.9nM
	A549	10nM	P<0.01	22.1nM
Etoposide (0-5μM)	MVEC	0.16μM	P<0.001	1.4μM
	SKMEL2	0.078μM	P<0.05	0.31μM
	MCF7	0.16μM	P<0.05	2.6μM
	A549	0.16μM	P<0.05	0.58μM
Carboplatin (0-500μM)	MVEC	250μM	P<0.01	Not reached
	SKMEL2	0.1μM	P<0.01	20μM
	MCF7	0.1μM	P<0.01	35μM
	A549	50μM	P<0.01	72μM

*MVEC – microvascular endothelial cell line; SKMEL2 – melanoma cell line; MCF7 – breast cancer cell line; A549 – lung cancer cell line. All results based on 3 independent experiments carried out in a minimum of triplicate. Agents in red   are those which are specifically anti-angiogenic or molecularly-targeted and therefore dose-ranges   are based on the clinical dose or previous phase I data. Agents in black are cytotoxic agents, where dose ranges were chosen based on less than 33% of the phase I MTD data (as per metronomic) and also using previous *in vitro* MTT data as a guide.

3.3 Discussion

The results have show that all five of the chosen orally bioavailable chemotherapeutic agents, each from a different class of compound, possess anti-endothelial properties when given in an ECS. Carboplatin however, seems to possess less of an anti-endothelial effect than the other four. The results are discussed in detail in this section. The three chosen specific agents, as expected, exhibit significant anti-endothelial effects at drug concentrations well within their normal clinical dose range, and each of their chemosensitvity assays acts as a baseline against which to compare potential synergistic effects.

Single anti-angiogenic agents

Sorafenib has received much attention in recent years and has shown promising results in Phase I-III clinical trials both as single agent and when used in combination, particularly in renal and lung cancer and malignant melanoma (Wilhelm S *et al* 2004; Clark J *et al* 2005; Mross K *et al* 2006). The clinical oral dose is 200 - 400mg twice daily, giving plasma concentration of 2.9mg/l. This is equivalent to 5000nM and as shown by our *in vitro* data, is also the dose which induces a significant anti-endothelial effect in the MVEC model. The fact that our *in vitro* dose-escalation data corresponds to the clinically safe dose in phase I trials, adds weight to our findings and to the use of this model. 5000nM was therefore the dose chosen to be carried forward into combination studies.

Combretastatin

This agent has shown promising results in phase I and II trials and is currently in

phase III combinatorial trials for use in thyroid cancer. In a phase I trial of CA4P on a 5 day schedule, with dose levels ranging from $6mg/m^2$ to $75mg/m^2$, the MTD was found to be $65mg/m^2$ (Stevenson J *et al* 2003). As previously stated, *in vivo* studies showed an anti-vascular effects of CA4P seen at concentrations less than one-tenth of the MTD (Dark G *et al* 1997). Results as shown in **figure 3.5**, indicate that CA4P caused significant MVEC inhibition at concentrations as low as 2.5nM ($p<0.01$). CA4P shows a concentration dependent effect, with a reduction in endothelial and tumour cell numbers, although the tumour cells were inherently more resistant than the MVECs. A dose of 5nM was chosen to be carried forward into combinatorial studies, as this dose caused statistically significant MVEC inhibition with a p value of <0.001, and is well within the clinical dose range.

Bortezomib

This non-specific anti-angiogenic agent has also shown promising results in clinical trials to date. In this study, significant MVEC inhibition was seen at concentrations as low as 1.56nM ($p<0.01$), less than 25% of the translated clinical dose. However, as expected, neoplastic cells were very susceptible to proteosome inhibition since its mechanism of action results in dysregulation of the cell cycle. The three cancer cell lines all reached their IC_{50} value in the dose range selected, corresponding to concentrations significantly smaller than the clinical dose.

Since Bortezomib was used here for comparative purposes, and is not a true anti-angiogenic agent, it was not carried forward for use in further combination studies.

Single chemotherapeutic agents

The five agents studied all showed promising anti-endothelial effects when given in

extended dosing schedules. IC_{50} values were reached for all agents, except carboplatin, well within the MTD and the arbritrary 33% MTD assigned to metronomic dosing.

Temozolomide has already been used in clinical extended-dosing schedules, showing promising results in treating brain and skin malignancies. In this study, it demonstrated significant anti-proliferative effects on MVECs at 10µM which equates to less than 15% of MTD (p<0.001). Other *in vitro* studies on HUVECs showed temozolomide to cause inhibition at doses as low as 2.5µM (1-5% of MTD, p<0.001) (Lam T *et al* 2007), at 5µM (differentiation) and at 50µM (proliferation) (Kurzen H *et al* 2003). The HUVEC defined 'metronomic' dose in these two examples is lower than that seen in the MVEC model. As previously explained in chapter 2, MVECs are more akin to tumour vasculature, having a greater degree of contact inhibition and relevant surface receptors than HUVECs, which may explain the difference in results and their slightly increased resistance.

In a Phase I trial for treatment of advanced glioma temozolomide was assessed for its potential in an extended dosing schedule. A 7-week daily oral schedule (75mg/m^2/day for 7 weeks – 50% MTD) was compared with a traditional 5-day regimen (200mg/m^2/day repeated every 28 days) (Brock C *et al* 1998). Overall response rates were similar, but the extended dosing schedule resulted in a lower incidence of toxicities and a 2.1-fold greater drug exposure over a 4-week period, showing its potential for use in extended dosing schedules.

Paclitaxel demonstrated an IC_{50} of 1.25nM for MVECs, which was also higher than

that of the HUVEC models of Bocci et al and Lam et al (Bocci G *et al* 2002; Lam T *et al* 2007) which showed IC_{50} values of 96pM and 228pM respectively. Previous pharmacokinetic studies involving paclitaxel have shown that 6 hour infusions and 24 hour infusions, each administered once every 3 weeks, resulted in an MTD of $250mg/m^2$ and corresponding C_{max} values of 13µM and 1µM respectively (Rowinsky EK *et al* 1992). The data suggests that paclitaxel may be used at less than 15% MTD as part of a combined 'metronomic' protocol to induce a significant anti-endothelial response. Bocci et al demonstrated that ECS with several cytotoxic agents including paclitaxel caused significant apopotosis in endothelial cells after 6 days of treatment (Bocci G *et al* 2002). These results provide evidence to justify the use of paclitaxel in metronomic schedules.

Vinorelbine demonstrated an IC_{50} of 16.9nM for MVECs, although significant anti-endothelial effects were displayed at 1nM ($p<0.001$) correlating to less than 10% MTD. It also demonstrated anti-melanoma effects, with significant inhibition demonstrated in the SK-MEL-2 cell line at 1nM ($p<0.05$). Previous *in vitro* studies have shown that another vinca alkaloid, vinblastine, possesses anti-angiogenic properties when applied at very low, non-toxic doses of 0.25-1pM in the HUVEC model (Vacca A *et al* 1999). Along side this, further *in vitro* studies in human melanoma cell lines have reiterated this finding, where by four vinca alkaloids including vinorelbine were found to induce IC_{50} values at very low doses ranging from 1pM to 10nM (Photiou A *et al* 1992). Pharmocokinetic studies involving vinorelbine compared a 20 minute intravenous infusion of $25mg/m^2$with gel capsules at a dose of $80mg/m^2$. Efficacy and safety profiles were similar for both routes, with C_{max} values of 133.4 ±42.3ng ml^{-1} (Marty M *et al* 2001; Whitehead RP *et al* 2004).

Etoposide demonstrated an IC_{50} of 1.4µM for MVECs, although the first signs of endothelial inhibition occurred at 0.078µM ($p<0.01$), corresponding to less than 10% of the MTD (Sessa C *et al* 1995). The SK-MEL-2 carcinoma cell line also showed significant inhibition to etoposide at 0.078µM ($p<0.01$). Both phase I and II trials have studied the effect of the orally bioavailable etoposide, particularly in ovarian cancer, but not as yet in combinatorial regimens (Markman M *et al* 1992; Baur M *et al* 2005). Previous pharmacokinetic studies illustrated that oral administration of 100mg/m^2 and 150mg/m^2 resulted in C_{max} values of 4.45 and 2.87µg/ml respectively (Simon G *et al* 2006). Several phase II studies using 50mg/m^2 etoposide once or twice daily for 14-21 days on cancer patients who were previously resistant to standard dosing and extended-scheduling demonstrated a positive response to this chronic regimen (Markman M *et al* 1992).

Carboplatin did not show significant endothelial inhibition until a dose of 250µM ($p<0.01$) and therefore did not reach IC_{50} value for MVECs in the dose range selected. This agent was therefore not taken forward for use in combination dosing schedules. Phase II and III trials have shown promising data when administering Carboplatin in low-dose schedules, but only when combined with other cytotoxics or radiotherapy (Jeremic B *et al* 1996). This was in the treatment of non-small cell lung cancer, and the recommended dose was 35mg/m^2, with an MTD of 40mg/m^2.

Estramustine is a cytotoxic drug presently licensed for use in the treatment of hormone-refractory prostatic carcinoma. It is a stable conjugate of oestradiol and normustine, and the clinically used form comes as prodrug. It is thought to disrupt

microtubule dynamics spindle, and acts at a similar binding site to paclitaxel and vinblastine (Laing N 1 *et al* 997). It has been previously been studied for its anti-endothelial properties in the HUVEC model (Lam T *et al* 2007) – suggesting inhibitory effects at 1-3μM (p<0.001) which is 50 times below its cytotoxic range.

Estramustine is the agent used in combination with Temozolomide in the clinical part of this study (chapter 5), and would ideally have made up the sixth chemotherapeutic agent. Unfortunately, since previous work carried out (Maraveyas A *et al* 2005; Lam T *et al* 2007), the pure substance is no longer available, despite efforts to source this from a number of alternative international suppliers. It will however be discussed in the clinical context.

All these agents described have good oral bioavailability and are capable of achieving anti-endothelial effects at doses well below MTD, holding potential in future metronomic schedules. Their anti-angiogenic response *in vivo* will be difficult to assess, and will rely on the development of accurate surrogate markers of tumour vasculature. Certainly, some clinical results so far indicate that prolonged oral regimens can improve drug therapeutic index, along with the benefits of reduced cytotoxic effects (Markman M *et al* 1992; Brock C *et al* 1998; Gasparini *et al* 2001; Whitehead RP *et al* 2004).

Throughout this aspect of the study, it became apparent that within the 'empirical metronomic range' there were a number of doses with significant anti-endothelial effect. It was necessary to reach a decision on which dose to take further to combination regimens, taking into account the literature 'mantra' of 1- 33% and also

the given potential cumulative doses (when shifting from MTD to daily dosing) a patient could receive. It was therefore decided that the first dose level with highly significant (p<0.001) MTT inhibition of MVECs compared to baseline would be taken forward to combination, provided it did not exceed 15% of MTD (as expected from the published literature range). It is accepted that this in itself is an arbitrary method of establishing a dosing level, but in this respect, there was no guidance in the literature. There is also the concern that many of the known data used as reference values were based on conventional pharmacokinetics. Conventionally drug schedules are bi-weekly, weekly, or every three or four weeks. Taking a dose as high as 33% of a drug that is normally given (for example) on a 3 weekly basis, could result in extremely high levels of cumulative dosing if this was extrapolated to a daily dosing schedule. With this in mind, the criteria were set to look for the first MTT assay with a reproducible highly significant result. If extrapolation from this dose kept the total potential daily exposure in a theoretical clinical setting to below 15% of the MTD dose, it was assumed that this would be a reasonable metronomic dose to do further experiments with.

In spite of the limitations of an *in vitro* model such as this, this study yielded results which compare favourably with other published works on *in vitro* dosing of metronomic chemotherapy. The aims of this part of the study, as laid out in section 3.1, have been met. Ideally, this study should be followed up using other assays or angiogenesis models, including those that more closely mimic tumour vasculature in 3-dimensional frameworks (Akhtar N *et al* 2002), and potentially in animal studies. Given the time and financial constraints of this piece of work, neither of these routes would have been visable to follow. It is hoped however, that further work can develop

from this study. Using one cell line for each cancer type studied was also a limitation of this work. However, this is a basic model and in order to improve statistical significance and the quality of the data, each drug concentration for each cell line was tested in sextuplicate. The MVECs, despite again being of from one cell line, are representative of endothelium which are common to all cancers and vasculature. It is hoped that the use of microvascular cells in this study, which more closely resemble tumour vasculature than large endothelial cells e.g HUVECs, will add weight to our findings. The limitations of *in vitro* work must however always be remembered in this case.

Chapter 4 will describe the results of the combinations of sorafenib and combretastatin with the chemotherapeutic agents. As mentioned earlier in this discussion, Bortezomib will not brought forward to test in the combination regimens since, although it is a molecularly-targetted agent, it was not designed to specifically target tumour vasculature, and was purely used for comparative purposes here.

The approach taken in this study does not further our understanding of the mechanisms of action of these agents, nor does it replace or even reflect the clinical setting (Maraveyas A *et al* 2005). It is however a potential framework, which is easily reproducible and reliable on which to base further *in vivo* studies and from which to initiate phase I anti-angiogenic trials.

Chapter 4

Chemotherapeutic and anti-angiogenic agents given in an extended-dosing schedule in combination using the *in vitro* MVEC model.

4.1 Aims and Introduction

The aims of the second part of the *in vitro* study described in this chapter are:

1. Using the *in vitro* MVEC model, to test the potential synergistic effects of combining the multi-kinase inhibitor, sorafenib, with the chemotherapeutic agents selected for this study (see chapter 3). This was using the established 'metronomic' dose of each agent, as described in chapter 3.

2. Using the *in vitro* MVEC model, to test the potential synergistic effects of the vascular-targetting agent, combretastatin with the chemotherapeutic agents selected for this study (see chapter 3). This was using the established 'metronomic' dose of each agent, as described in chapter 3.

3. To determine if an additive inhibitory effect on growth was seen in any of the cell lines, when comparing single-agent data to the combination regimens and to rule out combinations that show little or no synergy.

All clinical studies to date looking at metronomic scheduling confirm its excellent safety profile (see section 1.3.2). With few exceptions (Krzyzanowska M *et al* 2007), results of these studies have been considered worthy of further clinical evaluation. The published metronomic protocols are commonly complemented with antiangiogenic agents (see section 1.3.3), or agents with antiangiogenic properties attributed to them. These have included cyclooxygenase-2 (COX-2) inhibitors, glitazones, thalidomide and bevacizumab (Hau P *et al* 2007; Kesari S *et al* 2007;

Garcia A *et al* 2008).

This chapter shows the results of combination schedules of both sorafenib and combretastatin with the traditional chemotherapeutic agents given at 'metronomic' dose, as determined in chapter 3. This may provide some support to clinical evidence of useful combinations, and also potentially provide a starting point for designs of *in vivo* or phase I combinatorial studies in this confusing era of multi-targeted therapy.

4.1.1 Efficacy of metronomic scheduling of cytotoxic drugs in combination with anti-angiogenic agents: trial evidence to date

There have been numerous studies in recent years investigating the efficacy of combination regimens, with metronomic and antiangiogenic being the key goals of these treatment schedules.

The most interesting results to date have been achieved when metronomic chemotherapy was combined with bevacizumab (Burstein H *et al* 2005; Rocca A *et al* 2007; Garcia A *et al* 2008). Other important studies have included the use of metronomic cyclophosphamide (Lord R *et al* 2007) and cyclophosphamide with methotrexate and trastuzumab – a recombinant humanised anti-erbB2/HER-2 monoclonal antibody (Orlando L *et al* 2006; Lord R *et al* 2007). This regimen was shown to have some efficacy and minimal toxicity in advanced breast cancer patients, although a larger trial is required to confirm this (Bocci G *et al* 2005; Orlando L *et al* 2006).

Importantly, many of the recent combinatorial clinical studies do not pose

justification or rationale for the chosen optimal 'antiangiogenic' dose for the cytotoxic agent, and empiricism seems yet again to be the basis of dose-choice. With respect to dosing, the analysis of circulating endothelial (progenitor) cells or intra-patient dose-escalation have been proposed (Mancusco P *et al* 2006). Further refinement of this is required, along with better tools to monitor the antiangiogenic effects of metronomic regimens.

The choice of combination partners can be guided by the safety profile of such agents, taking into account the risk of pharmacokinetic interference, ease of administration and cost. Also, individual patient requirements need to be taken into account to determine which are most likely to benefit from combination metronomic schedules e.g. adjuvant versus palliative or bulky versus residual disease.

4.1.2 Rationale for selection of drug combinations for this *in vitro* model

For the purposes of the combination studies, sorafenib is the only specific antiangiogenic used, given its known mechanism of action – as previously described in chapter 3. In the single agent data, sorafenib showed significant anti-endothelial effects within its clinical dose range, as anticipated. Combretastatin is used as the other main combinatorial agent due to the general use and development of this drug in the antiangiogenic arena and the general 'acceptance' that it classifies as an antiangiogenic rather than a cytotoxic However the mechanism of action of combretastatin remains far from clear, and the metronomic dose for this agent was therefore established prior to combinatorial studies. Both sorafenib and combretastatin have been studied in combination regimens (Amaravadi RK *et al* 2007; Eisen T *et al* 2007; Yeung S *et al* 2007; Nathan P *et al* 2008).

As discussed in section 3.3, the choice of cytotoxic agents taken forward into combinatorial schedules for this study was based on the significant anti-endothelial effects seen by chosen agents at doses well below 33% of MTD (see section 3.3. and table 2.1). These chosen agents in clinical practice are almost all licenced for use as oral agents (except for paclitaxel), with known oral bioavailablity profiles and well described toxicity profiles. This study could therefore provide valuable preclinical evidence for potential synergistic regimens that can be taken on to further studies.

4.2 Results

4.2.1 Determination of *in vitro* synergy between cytotoxic agents and Sorafenib

4.2.1.1 Temozolomide

A synergistic effect of combined low dose Temozolomide (10µM) with Sorafenib (5µM) is seen in this model (see **Figure 4.1 & table 4.1**). Temozolomide at 10µM resulted in MVEC cell growth to 80.89% (±3.19) of control. Sorafenib alone at 5µM resulted in MVEC cell growth to 70.18% (±2.39) of control. However, in combination at these concentrations, there is a very significant (P<0.001) growth inhibition to 43.02% (±3.72) of control. Additive inhibitory activity was also seen in SK-MEL-2 cell growth, with 55.26% (±2.43) seen with Temozolomide alone, 40.09% (±1.76) seen with Sorafenib alone and again a very significant (p<0.001) 24.91% (±2.43) of control seen for the combination of both agents. Significant inhibitory response was also seen in the A549 cell line MCF7 cell lines, although was equivalent to the effect induced by sorafenib alone.

In summary, significant synergy between Temozolomide (at 15% MTD) and Sorafenib (at clinical dose) was demonstrated in MVEC and melanoma cell lines.

Figure 4.1: Growth response of cell lines: sorafenib and metronomically-dosed Temozolomide
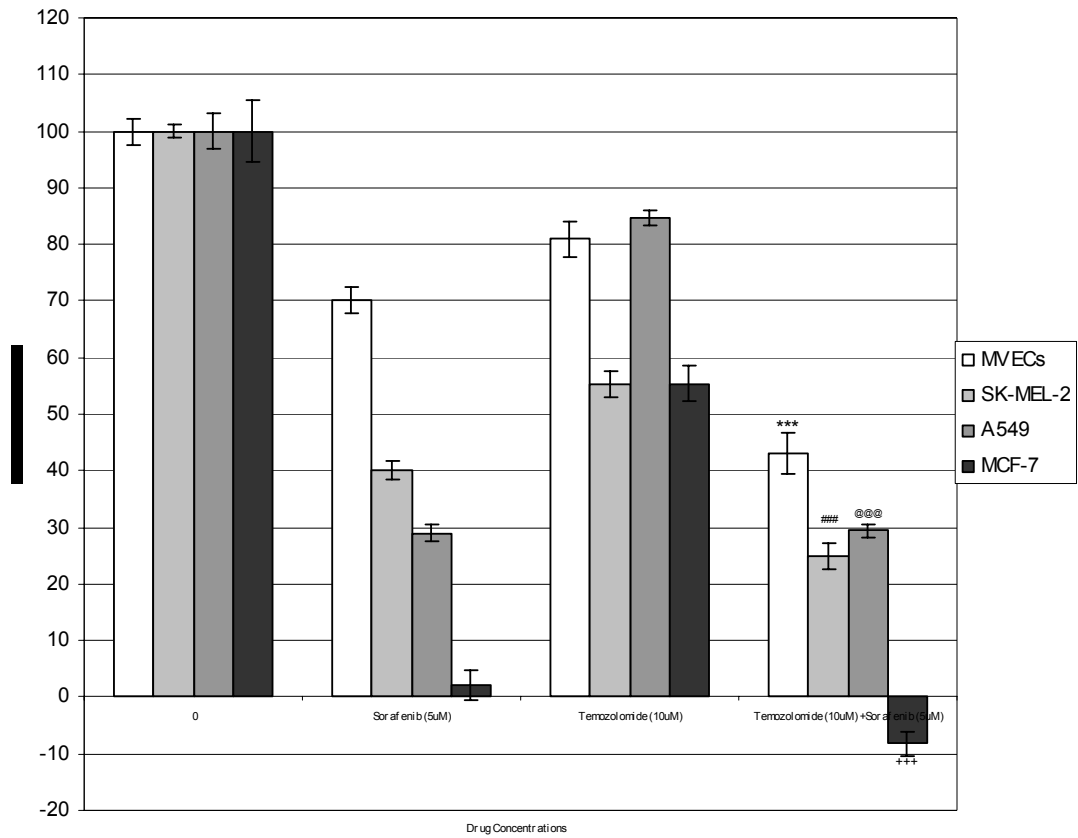
Growth of cell lines following 4 days of treatment with metronomically-dosed Temozolomide with sorafenib was determined by an MTT proliferation assay. All absorbance readings were performed using a spectrophotometric plate reader at 492nM and individual readings were background-subtracted. Readings at 4 hours post MTT addition were used as final readings for growth calculation. The growth of untreated cells was taken as 100% and the growth of treated populations of cells was expressed as a % of this. The histogram bars represent mean growth values +/- standard error and statistical significance of results is shown. The data shown are representative of at least 2 independent experiments, each conducted in triplicate.

4.2.1.2 Paclitaxel

A synergistic effect of combined low dose Paclitaxel (125pM) with Sorafenib (5µM) is seen in this model (see **Figure 4.2 & table 4.1**). When Paclitaxel and Sorafenib are combined at the predetermined doses (seen in Fig 2B), there is significant synergy (P<0.001) seen in both the MVEC and SK-MEL-2 cell lines. Paclitaxel (125pM) and Sorafenib (5µM) resulted in MVEC cell growth to 76.43% ±1.41 and 70.18%±2.39 of control growth respectively. However, in combination - at these concentrations, - 1.34% (±4) of control MVEC growth is seen. An additive inhibitory effect was also seen in the MCF7 cell line, with 2.13% (±2.52) seen with Sorafenib alone, 72.68% (±1.44) seen with Paclitaxel alone and -35.82% (±2.62) seen with combined agents. There was no significant additive response over above that seen with sorafenib alone in the A549 cell line.

In summary, significant synergy between Paclitaxel (at doses < 15% MTD – 125pM) and Sorafenib was demonstrated in both MVEC and SK-MEL-2 cell lines, and to a lesser extent, in the MCF7 cell line.

4.2.1.3 Vinorelbine

An additive anti-endothelial effect of combined low dose Vinorelbine (1nM) with Sorafenib (5µM) is seen in this model (see **Figure 4.3 and table 4.1**). When Vinorelbine and Sorafenib are combined at the predetermined doses (seen in Fig 2C), there is no significant anti-endothelial additive or synergistic response seen. However, synergistic activity was seen in the SK-MEL-2 and MCF7 cell lines, with 58.69% (±2.84) and 82.92% (±3.11) of control growth with Vinorelbine (1nM) given alone. However, in combination with Sorafenib, -61.67% (±4.74) and -249.41% (±7.31) of control growth was seen. There was no additive effect seen in the A549 cell line over and above that seen with sorafenib alone.

Figure 4.2: Growth response of cell lines: sorafenib and metronomically-dosed Paclitaxel
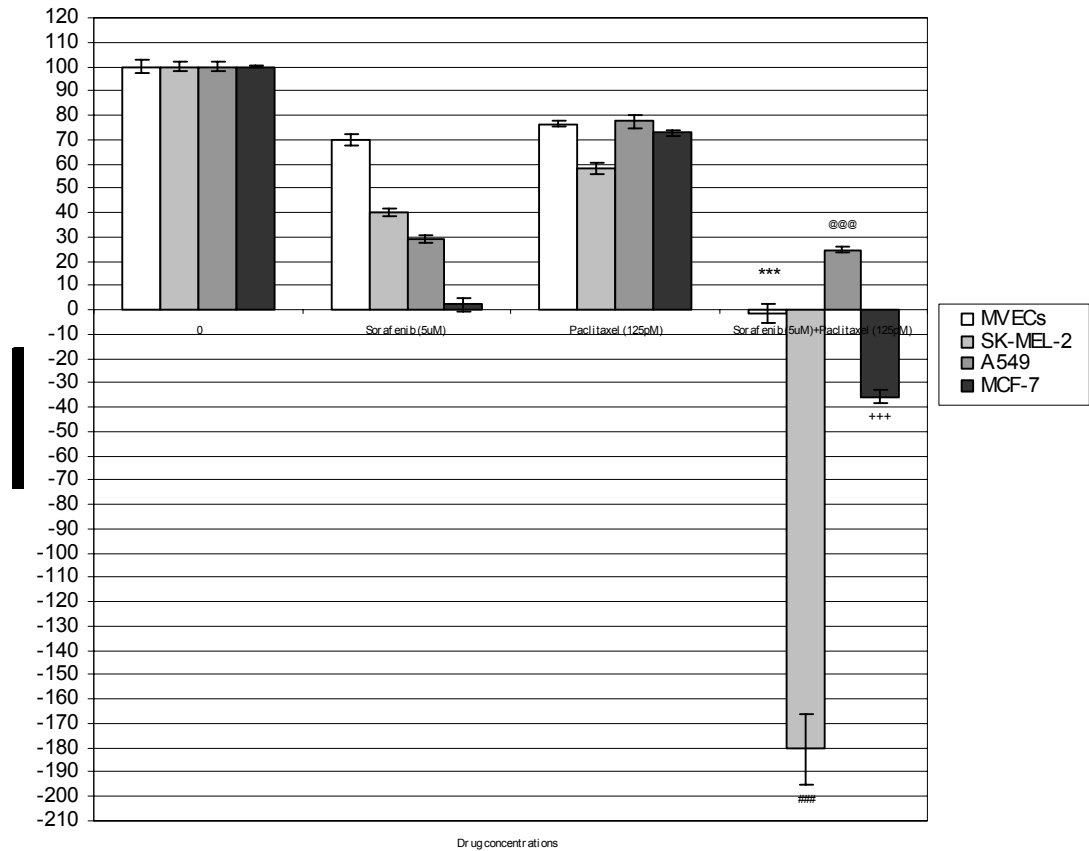
Growth of cell lines following 4 days of treatment with metronomically-dosed Paclitaxel with sorafenib was determined by an MTT proliferation assay. All absorbance readings were performed using a spectrophotometric plate reader at 492nM and individual readings were background-subtracted. Readings at 4 hours post MTT addition were used as final readings for growth calculation. The growth of untreated cells was taken as 100% and the growth of treated populations of cells was expressed as a % of this. The histogram bars represent mean growth values +/- standard error and statistical significance of results is shown. The data shown are representative of at least 2 independent experiments, each conducted in triplicate.

Figure 4.3: Growth response of cell lines: sorafenib and metronomically-dosed Vinorelbine
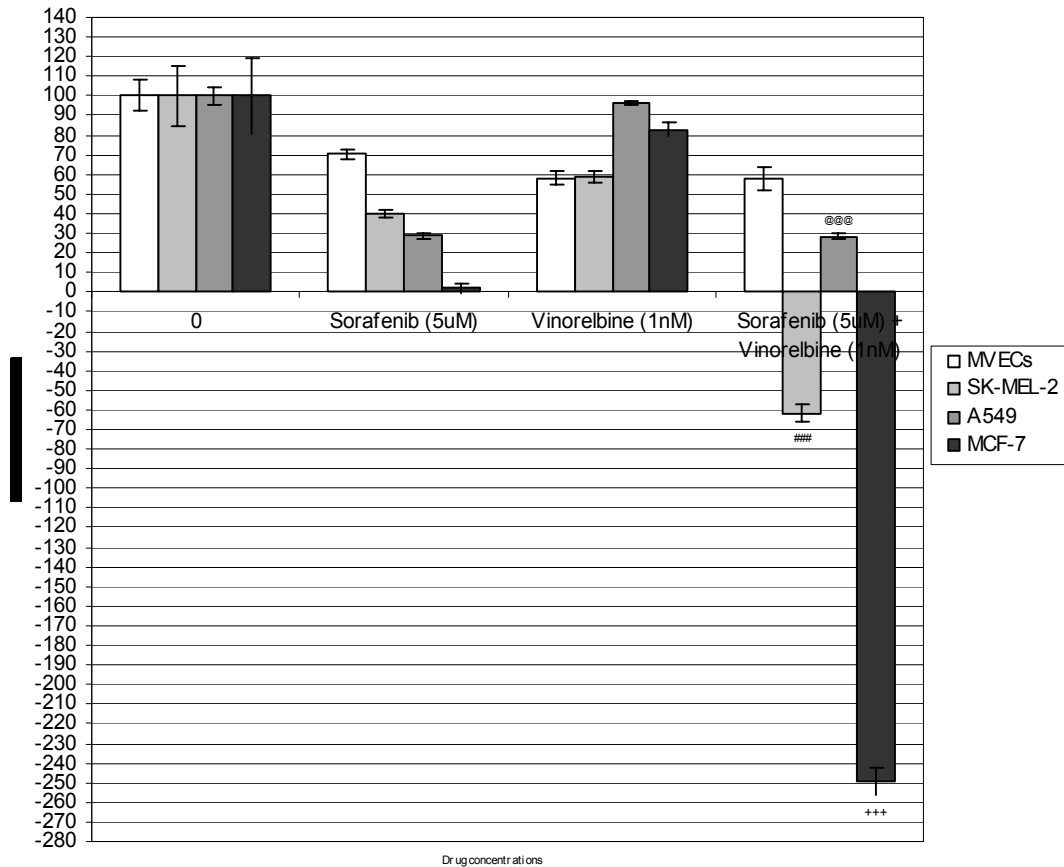
Growth of cell lines following 4 days of treatment with metronomically-dosed Vinorelbine with sorafenib was determined by an MTT proliferation assay. All absorbance readings were performed using a spectrophotometric plate reader at 492nM and individual readings were background-subtracted. Readings at 4 hours post MTT addition were used as final readings for growth calculation. The growth of untreated cells was taken as 100% and the growth of treated populations of cells was expressed as a % of this. The histogram bars represent mean growth values +/- standard error and statistical significance of results is shown. The data shown are representative of at least 2 independent experiments, each conducted in triplicate.

In summary, significant synergy between Vinorelbine (at doses < 10% MTD) and Sorafenib was demonstrated in SK-MEL-2 and MCF7 cell lines, but not in MVEC or A549 cell lines.

4.2.1.4 Etoposide

A synergistic effect of combined low dose Etoposide (0.16uM) with Sorafenib (5µM) is seen in this model (see **Figure 4.4 and table 4.1**). Significant anti-endothelial effects were observed when Etoposide and Sorafenib are combined (see Fig 2D). In this model, Etoposide alone resulted in MVEC cell growth to 65.52% ($\pm$2.57) and Sorafenib alone, in 70.18% ($\pm$2.39) of control. However, in combination, there is a significant (P<0.001) increase in growth inhibition to -40.09 ($\pm$5.13) of control. Additive inhibitory activity was also seen in the SK-MEL-2 cell line, with 61.55% ($\pm$1.4) with Etoposide alone, 40.09% ($\pm$1.76) with Sorafenib alone and in combination, a significant 3.84% ($\pm$1.28) of control growth. A549 and MCF7 cell lines showed no additive growth inhibition with this combination.

In summary, significant synergy between Etoposide (at doses < 10% MTD) and Sorafenib was demonstrated in MVEC and SK-MEL-2 cell lines.

Figure 4.4: Growth response of cell lines: sorafenib and metronomically-dosed Etoposide
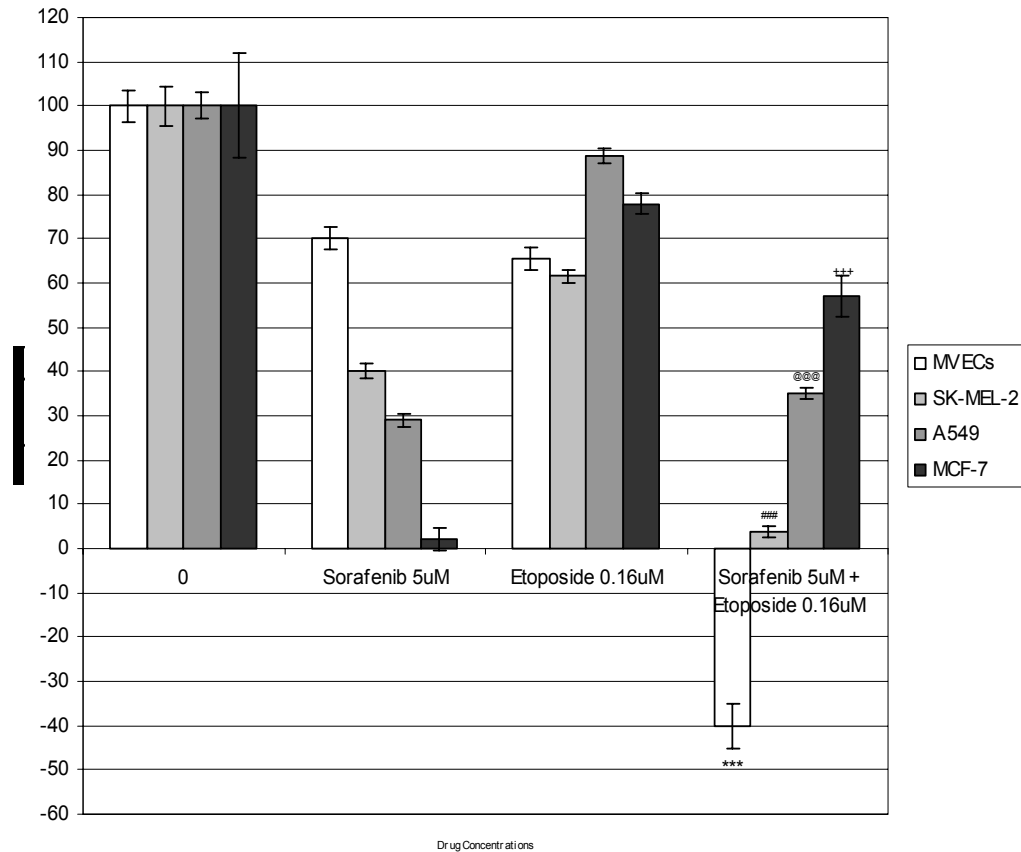
Growth of cell lines following 4 days of treatment with metronomically-dosed Etoposide with sorafenib was determined by an MTT proliferation assay. All absorbance readings were performed using a spectrophotometric plate reader at 492nM and individual readings were background-subtracted. Readings at 4 hours post MTT addition were used as final readings for growth calculation. The growth of untreated cells was taken as 100% and the growth of treated populations of cells was expressed as a % of this. The histogram bars represent mean growth values +/- standard error and statistical significance of results is shown. The data shown are representative of at least 2 independent experiments, each conducted in triplicate.

**4.2.2 Determination of *in vitro* synergy between cytotoxic agents and Combretastatin**

4.2.2.1 Temozolomide

MVEC growth was inhibited by 80.89% of control growth ($\pm$3.19) at the metronomic dose 10μM of single agent Temozolomide ($p<0.001$). When combined with combretastatin, no synergistic or additive effect is seen in any of the cell lines (see **Figure 4.5**).

4.2.2.2 Paclitaxel

MVEC growth was inhibited by 23.6% when treated with 125pM Paclitaxel alone, whilst addition of 5nM CA4P resulted in 60% endothelial cell inhibition compared to the untreated cell population ($p<0.001$). Therefore the combination schedule demonstrated a significant enhancement of growth inhibition of MVECs compared to the single chemotherapeutic schedule. However, treatment of MVECs with 5nM CA4P alone had an improved response compared to the combinational schedule achieving a percentage endothelial cell inhibition of 66.5%. Paclitaxel therefore had an inhibitory effect on the CA4P growth inhibition. Addition of 5nM CA4P to 125pM Paclitaxel resulted in a statistically significant antagonistic effect in the SK-MEL-2 ($p<0.001$), A549 ($p<0.001$) and MCF7 ($p<0.001$) cell lines compared to 125pM of paclitaxel alone (see **Figure 3.8**). Single paclitaxel treatment at 125pM resulted in 42.2% SK-MEL-2, 22.4% A549 and 27.3% MCF-7 cell inhibition (compared to the corresponding untreated cell population), whilst in combination with 5nM CA4P, the percent cell inhibition in the SKMEL-2, A549 and MCF-7 cell lines was 9.0%, 10.4%

Figure 4.5: Growth response of cell lines: combretastatin and metronomically-dosed Temozolomide
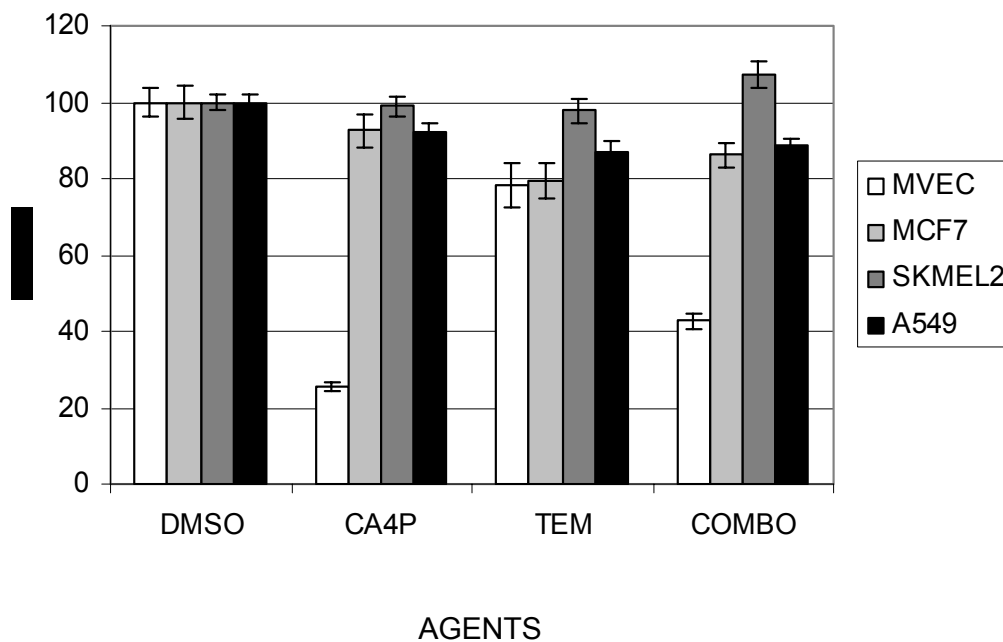
Growth of cell lines following 4 days of treatment with metronomically-dosed Temozolomide with CA4P was determined by an MTT proliferation assay. All absorbance readings were performed using a spectrophotometric plate reader at 492nM and individual readings were background-subtracted. Readings at 4 hours post MTT addition were used as final readings for growth calculation. The growth of untreated cells was taken as 100% and the growth of treated populations of cells was expressed as a % of this. The histogram bars represent mean growth values +/- standard error and statistical significance of results is shown. The data shown are representative of at least 2 independent experiments, each conducted in triplicate.

and 18.6% respectively (see **Figure 4.6 and table 4.1**). In summary, this *in vitro*

combination showed no evidence of increased anti-endothelial activity than CA4P as

a single agent, but did show some favourable anti-malignant cell responses.

4.2.2.3 Vinorelbine

Treatment of MVECs with 1nM vinorelbine alone caused 41.9% endothelial cell

inhibition, whilst addition of 5nM CA4P resulted in 64.2% endothelial cell inhibition

compared to the untreated MVEC population (see **Figure 4.7 and table 4.1**). The

combinational drug schedule therefore achieved significant enhanced growth

inhibition ($p<0.001$) compared to the single vinorelbine treatment. Again, treatment

of MVECs with 5nM CA4P alone had a better effect than the combination regime in

achieving endothelial cell inhibition. Addition of 5nM CA4P to 1nM vinorelbine

resulted in a significant antagonistic effect in the SK-MEL-2 and MCF-7 cell lines

($p<0.001$) compared to vinorelbine treatment alone. Single vinorelbine treatment

resulted in 41.3% SK-MEL-2 and 17.1% MCF7 cell inhibition (compared to

untreated cells) but in combination with 5nM CA4P, the SK-MEL-2 cell line

proliferated by 11.1% and the MCF-7 cell line was inhibited by 0.02% compared to

the corresponding untreated cell populations. For the A549 cell line, no significant

synergy was demonstrated between 5nM CA4P and 1nM vinorelbine compared to

1nM vinorelbine treatment alone.

In summary, antiendothelial effects with this combination were unremarkable, but

significant synergy was seen in the SK-MEL-2 and MCF7 cell lines.

4.2.2.4 Etoposide

When treated with 0.16µM etoposide alone, MVEC growth was inhibited by 34.5%

while addition of 5nM CA4P resulted in 66.5% endothelial cell inhibition compared

Figure 4.6: Growth response of cell lines: combretastatin and metronomically-dosed Paclitaxel
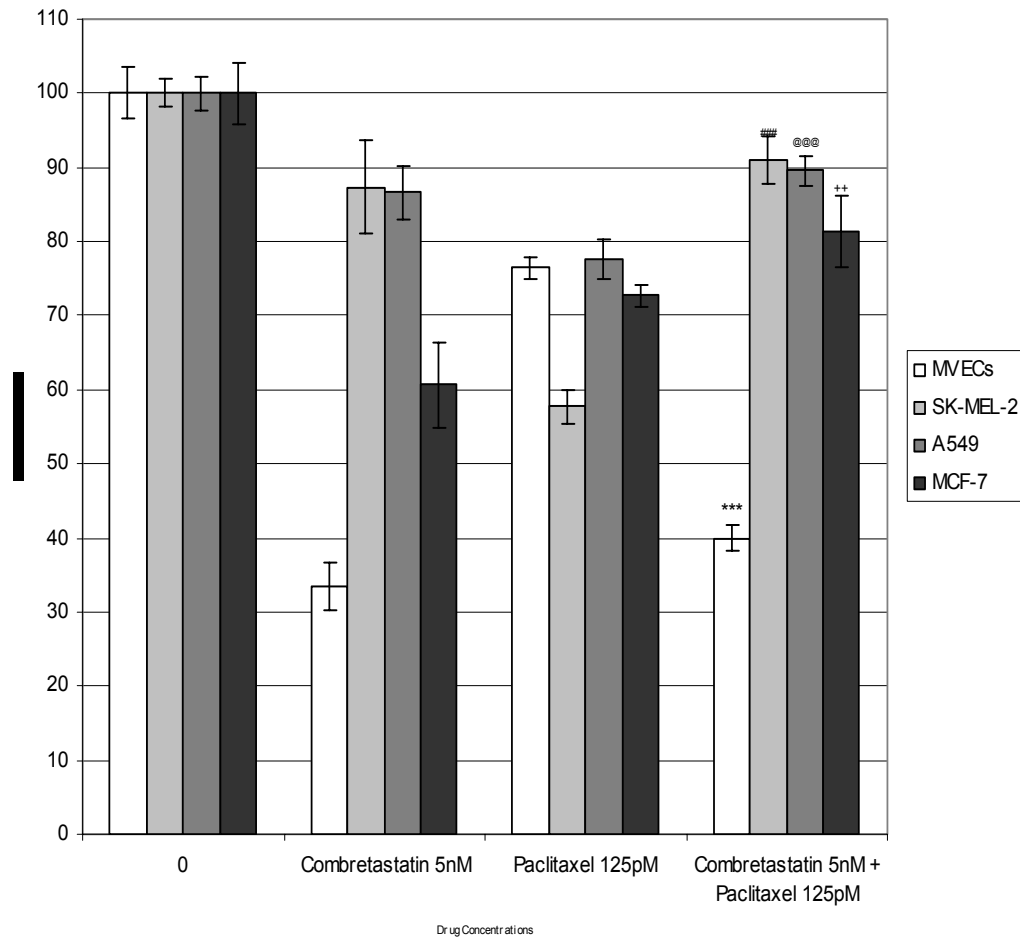
Growth of cell lines following 4 days of treatment with metronomically-dosed Paclitaxel with CA4P was determined by an MTT proliferation assay. All absorbance readings were performed using a spectrophotometric plate reader at 492nM and individual readings were background-subtracted. Readings at 4 hours post MTT addition were used as final readings for growth calculation. The growth of untreated cells was taken as 100% and the growth of treated populations of cells was expressed as a % of this. The histogram bars represent mean growth values +/- standard error and statistical significance of results is shown. The data shown are representative of at least 2 independent experiments, each conducted in triplicate.

Figure 4.7: Growth response of cell lines: combretastatin and metronomically-dosed Vinorelbine
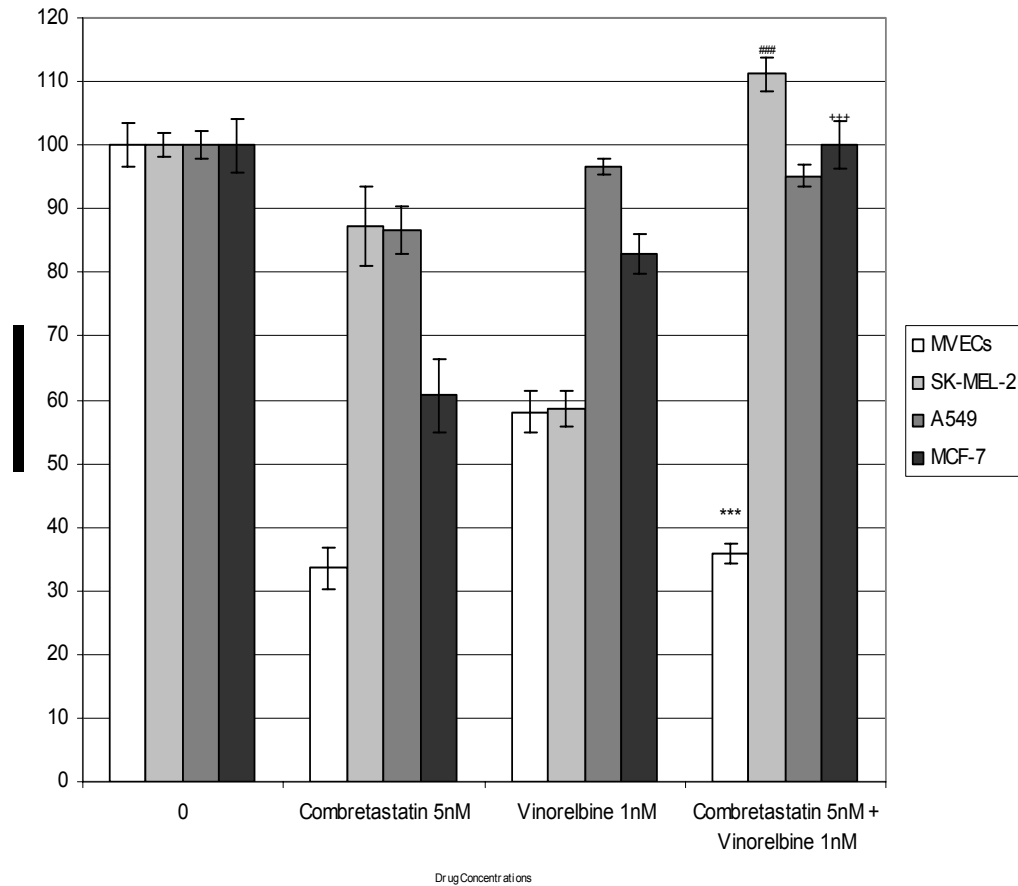
Growth of cell lines following 4 days of treatment with metronomically-dosed Vinorelbine with CA4P was determined by an MTT proliferation assay. All absorbance readings were performed using a spectrophotometric plate reader at 492nM and individual readings were background-subtracted. Readings at 4 hours post MTT addition were used as final readings for growth calculation. The growth of untreated cells was taken as 100% and the growth of treated populations of cells was expressed as a % of this. The histogram bars represent mean growth values +/- standard error and statistical significance of results is shown. The data shown are representative of at least 2 independent experiments, each conducted in triplicate.

to the untreated cell populations (see **Figure 4.8 and table 4.1**). Therefore, the combined drug schedule demonstrated a significant improvement in growth inhibition of MVECs ($p<0.001$) compared to the single chemotherapeutic schedule. Again, treatment of MVECs with 5nM CA4P alone had a greater effect than the combined schedule (compared to the untreated MVEC population). For the A549 cell line, a slight but significant improved growth inhibition ($p<0.05$) was observed in the combinational regime, achieving an MVEC inhibition of 17.1% compared to the untreated cell population, whilst single agents etoposide (0.16µM) and CA4P (5nM) achieved 11.2% and 13.4% cell inhibition, respectively (see Figs 3.10 and 3.5). No significant synergy or additive inhibitory activity was demonstrated between the combinational regime and single etoposide schedule for the MCF-7 cell line. However, addition of 5nM CA4P to 0.16µM etoposide resulted in a statistically significant antagonistic effect in the SK-MEL-2 cell line ($p<0.001$) compared to etoposide treatment alone. Single 0.16µM etoposide treatment alone achieved 38.5% SK-MEL-2 cell inhibition, single 5nM CA4P treatment achieved 11.3% SK-MEL-2 cell inhibition and the combinational schedule achieved SKMEL-2 cell inhibition of 12.6%. Therefore, the addition of 5nM CA4P to 0.16µM etoposide appears to have negated the cell inhibitory potential of etoposide in this particular cell line.

In summary, the use of this combination in the *in vitro* model described showed no significant synergistic activity in the cell lines used.

Figure 4.8: Growth response of cell lines: combretastatin and metronomically-dosed Etoposide
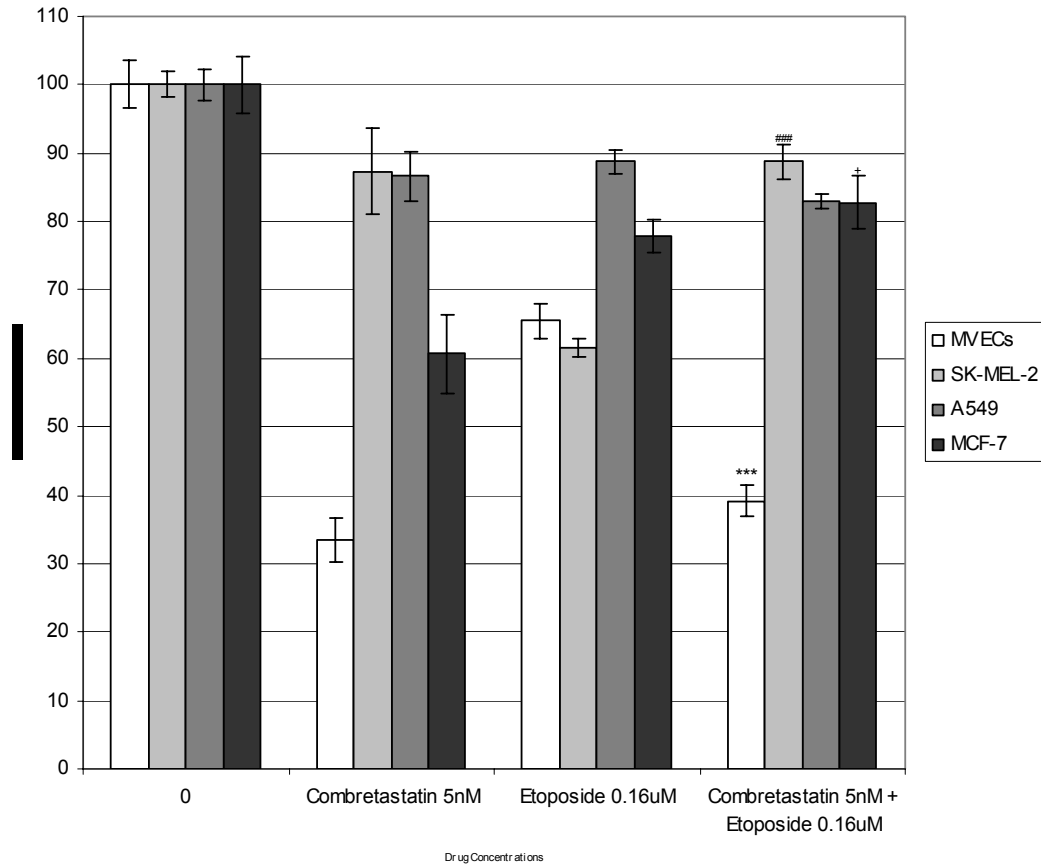
Growth of cell lines following 4 days of treatment with metronomically-dosed Etoposide with CA4P was determined by an MTT proliferation assay. All absorbance readings were performed using a spectrophotometric plate reader at 492nM and individual readings were background-subtracted. Readings at 4 hours post MTT addition were used as final readings for growth calculation. The growth of untreated cells was taken as 100% and the growth of treated populations of cells was expressed as a % of this. The histogram bars represent mean growth values +/- standard error and statistical significance of results is shown. The data shown are representative of at least 2 independent experiments, each conducted in triplicate.

Table 4.1: Summary Table of results of combinations of antiangiogenics and cytotoxics given at metronomc dose in the in vitro model.

Agent Combination	Cell line	Single agent – cytotoxic (% growth inhibition +/- SE)	Single agent – antiangiogenic (% growth inhibition +/- SE)	Combination – (% growth inhibition +/- SE + p value)	Additive effect seen
Temozolomide (10μM) + Sorafenib (5μM)	MVEC	80.89+/-3.19	70.18+/-2.39	43.02+/-3.72 p<0.01	Yes
	SKMEL2	55.26+/-2.43	40.09+/-1.76	24.91+/-2.43 p<0.001	Yes
	MCF7	55.8+/-5.3	2.3+/-6.4	-8.3+/-4.1 p<0.01	Yes
	A549	No additive effect			
Paclitaxel (125pM) + Sorafenib (5μM)	MVEC	76.43+/-1.41	70.18+/-2.39	-1.34+/-4 p<0.001	Yes
	SKMEL2	58.2+/-2.2	40.09+/-1.76	-180+/-8.0 p<0.001	Yes
	MCF7	72.68+/-1.44	2.3+/-6.4	-35.82+/-2.62 p<0.01	Yes
	A549	No additive effect			
Vinorelbine (1nM) + Sorafenib (5μM)	MVEC	No additive effect			
	SKMEL2	58.69+/-2.84	40.09+/-1.76	-61.67+/-4.74	Yes
	MCF7	82.92+/-3.11	2.3+/-6.4	-149.41+/-7.31	Yes
	A549	No additive effect			
Etoposide (0.16μM) + Sorafenib (5μM)	MVEC	65.52+/-2.57	70.18+/-2.39	-40.09+/-5.13 p<0.001	Yes
	SKMEL2	61.55+/-1.4	40.09+/-1.76	3.84+/-1.28 p<0.001	Yes
	MCF7	No additive effect			
	A549	No additive effect			
All cytotoxic agents + CA4P (5nM)	MVEC	No additive effect seen with any of the cytotoxics			
	SKMEL2				
	MCF7				
	A549				

4.3. **Discussion**

This chapter has investigated the potential synergistic effects of extended continuous low dose chemotherapy in combinination with sorafenib and combretastatin.

The rationale behind combination regimens as they appear in clinical trials is currently wholly empirical. The underlying mechanisms of action of such combinations and whether synergistic action exists are poorly understood. One can speculate from the molecular knowledge of specific single agents' mechanism of action, and assume that certain agents are likely to be effective in specific tumours, but again, this is an empirical approach, showing little understanding of or justification for multi-targeted therapies (Maraveyas A *et al* 2005). Further studies are thus required which determine and potentially predict which agents may be synergistic, and for what reason at a molecular level. It is with this in mind, that the aim of this chapter was to assess the *in vitro* combination regimens for their efficacy.

For the **Temozolomide / Sorafenib** combination, significant synergistic enhancement of the inhibitory effect of these agents in both the MVEC and the SK-MEL-2 cell lines was found. The combination out-performed each single agent in these cell lines by an additional 37.87% and 30.35% (p<0.001) respectively (see **figure 4.1**). Temozolomide is an alkylating agent, and there is growing clinical evidence to support the use of alkylating agents, including cyclophosphamide, in metronomic combination regimens (Orlando L *et al* 2006; Eisen T *et al* 2007; Lord R *et al* 2007; Rocca A *et al* 2007; Garcia A *et al* 2008) . Further framework studies, as this one described, may be useful for agents similar in order to demonstrate the potential validity of current combinations and to potentially give clues to finding new

synergistic regimens.

As previously described, the scientific rationale for using Sorafenib in the treatment of melanoma is clear (Egberts F *et al* 2008). Single agent trials in melanoma were disappointing (Flaherty K *et al* 2005), although were very promising in other solid malignancies e.g. liver and renal cell carcinoma (Escudier B *et al* 2005; Llovet J *et al* 2007; Llovet J *et al* 2008). In melanoma, a two or even three-hit strategy will no doubt be more effective. Flaherty poses the hypothesis that the Temozolomide/Sorafenib duo work well together through BRaf inhibition and apoptotic pathways, but that ideally a second targeted agent, rather than a cytotoxic, would likely induce a more profound response (Tuma R 2006). This combination used in metastatic melanoma already holds promise in phase II clinical trials (Amaravadi RK *et al* 2007). When combined with the parent compound DTIC, Sorafenib has produced further encouraging improvements in progression free survival (McDermott D *et al* 2008). This randomised double-blind phase II study showed no difference in overall survival with the combination, but significant improvement in PFS rates at 6 and 9 months, and in TTP (median, 21.1 v 11.7 weeks). The regimen was also well tolerated with a manageable toxicity profile. In an even more recent phase II trial of temozolomide and sorafenib in advanced melanoma patients with or without brain metastases, an extended schedule of temozolomide showed a moderate (but not significant) difference in PFS compared to a standard dosing regimen (Amaravadi R *et al* 2009). Phase III results are needed to determine true clinical synergy.

With regards to the **Paclitaxel / Sorafenib** combination, over 70% synergistic

enhancement of the inhibitory effect of these agents was seen in MVEC, SK-MEL-2 and MCF7 cell lines (see **figure 4.2**). As previously stated, paclitaxel is a mitotic inhibitor which has been shown to induce anti-endothelial effects at concentrations lower than those required to inhibit tumour cell proliferation (Drevs J *et al* 2004; Albertsson P *et al* 2006). Its combination with Sorafenib in clinical trials has, to date, shown mixed results. In 2008, a phase III trial evaluating the benefit of adding sorafenib to the combination of carboplatin and paclitaxel for the treatment of non-small cell lung cancer, was halted due to a higher mortality being seen in the sorafenib group. Interestingly, our *in vitro* study also showed no synergistic activity with this combination in the A549 (lung cancer) cell line which was in fact the only cell line which did not show a positive response to the combination. Comparison between *in vitro* single cell line studies and clinical trials can only be observational and not translational, until intermediate studies can add weight to data, but it is nevertheless an interesting observation in this study. The Paclitaxel / Sorafenib combination is now also being studied in phase II trials for stage IV melanoma and metastatic breast cancer, with results awaited. Paclitaxel has shown a modest effect in the treatment of metastatic melanoma to date, but is currently only used as a second-line therapy and in combination with carboplatin (Rao R *et al* 2006). Results of phase III trials of these agents used as second-line treatment in melanoma, showed no improvement in PFS or ORR with the addition of sorafenib (Agarwala S *et al* 2007). *In vitro* evidence may well have provided a sound basis for these combinations prior to them reaching the *in vivo* setting.Results of this same triple combination are currently awaited from an active trial in patients with stage IV melanoma of the eye.

The **Vinorelbine / Sorafenib** combination showed no significant enhanced

endothelial response, but did show over 100% enhanced response to single agents with the melanoma and breast cancer cell lines (see **figure 4.3**). Clinical trials to date in vinorelbine-based melanoma treatment have been mainly focussed on single agent or multi-chemotherapeutics, without an anti-angiogenic agent. So far, results have been disappointing (Retsas S *et al* 1996; Whitehead R *et al* 2004). Anti-angiogenic style phase II study results are awaited. In other solid malignancies, such as breast cancer, phase II phase II trials of vinorelbine in combination with antiangiogenics i.e. trastuzumab show promise (Chollet P *et al* 2007). Interestingly, these early trials using anti-angiogenic agents often involve conventional dosing of chemotherapeutics, which as previously explained, would seem to negate the efficacy of such an approach. Since our *in vitro* model showed no enhanced anti-endothelial effect with this combination, further studies comparing conventional and metronomic schedules may provide further information on agent and dose synergy. This emphasises the importance of a screening tool to determine which chemotherapeutics may be suitable for the metronomic response.

The **Etoposide / Sorafenib** combination showed a significantly enhanced response compared to single agents in both the MVEC and SK-MEL-2 cell lines by over 55% (see **figure 4.4**). Of all the sorafenib-chemotherapy combinations, this combination showed the greatest anti-endothelial synergistic effect. This was greater than the effect seen in both the temozolomide and paclitaxel combinations. This suggests that the combination has a very active anti-endothelial effect, and would potentially warrant future study in both the *in vivo* and clinical setting. In designing such future studies, it would be important to start where etoposide has known phase III data (eg small cell lung, ovarian and prostate cancer) although, as with all anti-angiogenic

clinical trials, monitoring of efficacy and response remains a challenge (Artal-Cortes A *et al* 2004). Chapter 5 will discuss the potential use of CECs and CEPs in monitoring response in anti-angiogenic, metronomic regimens. The findings from the melanoma cell line data could be compared to an earlier phase II etoposide / cisplatin trial in patients with melanoma that showed no additional clinical efficacy (Eton O *et al* 1991). It may be that an *in vitro* screening tool for combination regimens may help to eliminate ineffectual combinations from heading to the clinical stage.

The effects of combining Combretastatin, a non-specific anti-angiogenic agent, were not as successful as that observed with a specifically targeted agent such as Sorafenib. These results concur with the findings of Lam *et al* who compared the effects of combining a specific anti-VEGFR-2 monoclonal antibody to a non-specific farnesyl transferase inhibitor in a combinational schedule with low dose cytotoxics in an *in vitro* HUVEC model (Lam T *et al* 2007). With respect to the effects of combinational therapy involving CA4-P and the low dose cytotoxic agents on the 3 carcinoma cell lines, in the majority of cases, an antagonoistic effect was seen in comparison to the single therapy schedules (see **figures 4.5-4.8**). In particular, CA4-P in combination with vinorelbine resulted in 11.1% proliferation (compared to untreated cell populations) in the SK-MEL-2 cell line, whilst both single agents demonstrated anti-proliferative effects upon this cell line (see **figures 3.5 and 3.9**). Therefore, although CA4-P combined with low dose chemotherapy resulted in some anti-endothelial effects, the adverse effects demonstrated in the cancer cell lines suggests that CA4-P may have reduced the efficacy of the cytotoxics, making the combinations unsuitable in this *in vitro* model.

Chemotherapeutic activity has, in the past, been shown to diminish with decreased tissue oxygenation and blood flow (Horsman M *et al* 1991). Since CA4-P is known to cause tumour vascular disruption, it has the potential to disrupt or even diminish the efficacy of cytotoxics (Dark G *et al* 1997). With an *in vitro* model, it is impossible to assess these pathophysiological mechanisms of action, and therefore further *in vivo* work would be required to specifically look at whether vascular disruption truly limits chemotherapeutic efficacy. There is pre-clinical evidence however to show excellent synergy between carboplatin and CA4-P in rodent models, but this combination was terminated in a phase I clinical trial due to early dose-limiting toxicity (Bilenker J *et al* 2005). Through these combination studies, we have excluded carboplatin as it was shown in the single agent studies to be a drug with no obvious metronomic profile. Moreover we have failed to find any combretastatin chemotherapy combination better than combretastatin alone. The limitations of the *in vitro* study model have however prevented the assessment of combretastatin's mechanism of action on vasculature, and without more reliable *in* vivo or clinical data, the combinations can not be ruled out as being inappropriate. However, if further pre-clinical work, based on these findings, suggested similar futility from such combinations, it may prevent phase I trials, based on empiricism, from being undertaken.

The study has however shown that at least four sorafenib chemotherapy combinations have a synergistic antiendothelial and antiproliferative effect, of which the most striking was that of sorafenib and etoposide as discussed above. These results can provide a starting framework from which further *in vivo* and clinical studies can take place.

Although *in vitro* work will never fully translate to human studies, here we demonstrate a logical approach, using clinically relevant agents at achievable doses and in relevant combinations. In this potentially confusing era of multi-targeted combinational therapy, new agents are being developed almost every week. Oncologists require a framework or at least a justifiable starting point to select drug combinations and doses for patients with all malignancies (Maraveyas A *et al* 2005). The work in the last 2 chapters delineates one potential such approach, and may well aid the early elimination of ineffectual or even deleterious combinations. Since combinatorial options are huge, alternative, potentially more effectual options are plentiful, provided they are at least given the chance at an *in vitro* level first.

The other important issue here is that true antiangiogenic approaches are being used in only a handful of new combination trials (Glode L *et al* 2003; Gille J *et al* 2005; Colleoni M *et al* 2006; Kesari S *et al* 2007; Lord R *et al* 2007). Instead, many multi-agent trials are using conventionally-dosed chemotherapy - which as previously described is pro-angiogenic - with specific anti-angiogenics in the hope of achieving success (International Adis 2008; McDermott D *et al* 2008). These are often based on intuition and empirisicim rather than a true scientific justification for the regimen. It would seem counter-productive to pursue such proangiogenic-antiangiogenic combinations given the current molecular understanding of tumour biology. This reiterates and emphasises the urgency for screening tools and surrogate markers of angiogenesis.

Throughout the last two chapers, the limitations of using *in vitro* models such as MVECs or any other endothelial cell type to represent tumour endothelium have been

emphasised. As previously mentioned, we feel that there is a valid argument to use the MVEC model over the HUVEC model, since cells of microvascular origin more accurately reflect angiogenesis *in vivo* (Salcedo R *et al* 2000). However, neither model take into account the anatomical complexity of tumour-related vasculature, which includes other essential stromal and vessel-wall components.*In vivo* animal models can take into account the pharmacodynamic dimension that this model would not be able to provide. *In vitro* models will never truly represent the *in vivo* process, although *in vitro c*hemosensitivity assays do play an important role in the development of regimens and add weight to clinical trials (Browder T *et al* 2000; Bocci G *et al* 2002; Lam T *et al* 2007). *In vivo* studies looking at drug combinations in the future, could potentially use similar *in vitro* screening tools in order to have some evidence, although rudimentary, on which to base *in vivo* their work. Our work demonstrates that the effects of ECS can be significantly enhanced with addition of a specific agent that targets molecules involved in angiogenesis. It is a readily available, reproducible and technically simple *in vitro* screening system for drugs used in cancer treatment, either as single or combination agents.

This model does not supplant or obviate the need of *in vivo* work. It can however prioritise more promising regimens and in some cases demonstrate clearly antagonistic combinations that would not be justified to be studied further otherwise. The question of ethics of taking ineffective combinations to further study if at a cellular level they are actually shown to be antagonistic is clearly raised. With limited resources for further *in vivo* studies, our data would clearly prioritise which combinations should be preferred over others. For example from this work the combination of sorafenib and etoposide would be prioritised for further study in

relevant cancer settings.

Since there are an ever increasing number of traditional cytotoxic and molecularly-targeted agents, the task of categorising all agents is vast. However, it is important to produce patterns of synergistic responses between agents and eventually move forward to determine changes in down-stream proteins that are pertinent to specific agents. Analysis of the proteome may well prove to uncover unknown targets of both specific and non-specific agents, and also shed light on mechanisms of synergy or antagonistic effects (Rosenblatt K *et al* 2004). This may also provide further justification for tailor-made regimens designed for specific patients and tumours, aiming for maximum efficacy rather than maximum cytotoxicity.

In conclusion, further studies need to be undertaken to define the mechanisms of action of different anti-angiogenic agents, which cytotoxics are suitable for the metronomic approach and to determine true synergistic responses that may, in the future, be taken forward to the clinic. Cancer as a whole remains the second leading cause of death in the developed world. A cure may still be a distant hope, but the metronomic, multi-targeted approach has potential for producing a chronic disease state.

Chapter 5
Isolation of CECs and CEPs in patients with metastatic melanoma on metronomically-dosed chemotherapy versus conventionally-dosed chemotherapy.

5.1 Aims and Introduction

The aims of this chapter are:

1.   To test methodology and to optimise the technique of isolating CECs and CEPs from whole blood.

2.   To isolate CECs and CEPs from whole blood samples of patients recruited into the scientific sub-study (as described in chapter 2).

3.   To compare CEC and CEP levels between patients on metronomically-dosed and conventionally-dosed chemotherapy.

Angiogenesis is now known to be central to tumour biology. As described throughout previous chapters, it is the discovery of this that has led to the development of a multitude of molecularly-targeted anti-angiogenic agents. Accompanying this, there is an increasing demand for surrogate markers that reflect tumour vasculature and its response to treatment (Ruegg C *et al* 2003). The endpoints of classical anti-cancer trials no longer give sufficient discriminative powers in antiangiogenic trials. Currently, the National Cancer Institute recommends that RECIST (Response Evaluation Criteria in Solid Tumours) is used to discriminate response. This criteria involves the use of Xray, CT and MRI to measure target lesions, and despite attempts to improve this system using 3D technology, it remains an inadequate evaluation tool in the context of antiangiogenic trials (Therasse P *et al* 2009).

In the last decade, there have been over 100 molecules (e.g. VEGF, angiogenin, D-dimer) that have shown antiangiogenic activity in preclinical studies, with only a handful showing measurable anti-tumour activity in clinical trials (Bocci G *et al* 2004; Sandler A *et al* 2006; Motzer RJ *et al* 2007). **Table 5.1** shows a list of agents which have been shown to inhibit tumour angiogenesis (Horsman M *et al* 2010). Identification and validation of molecular and cellular markers remains essential, particularly in determining long-term efficacy of antiangiogenic regimens.

Two such markers, circulating endothelial cells and circulating endothelial progenitor cells (CECs and CEPs – see section 1.2.3 and 1.2.5), have been identified and continue to be validated as potentially useful markers of tumour vasculature activity (Raia V *et al* 2007). There is accumulating evidence from both pre-clinical and clinical studies that they play an important role in tumour growth and neovascularisation, as discussed in section 1.2.5 (Carmeliet P and Lutton 2001; Lyden D *et al* 2001; Rafii S *et al* 2002; Davis DW *et al* 2003; Beerepoot L *et al* 2004; Peters B *et al* 2005; Duda D *et al* 2006; Yu D *et al* 2007; Calleri A *et al* 2009). Several assays, based on manual or automated immunomagnetic isolation or flow cytometry, have been described for the detection and quantification of these cells to date. The different assays described are subject to errors in sampling, preparation and analysis (Strijbos M *et al* 2008; Michael A *et al* 2010). Although a consensus on the optimal technique is still lacking, the number of studies assessing CECs and CEPs in cancer patients is rapidly expanding, and more recently, showing varying trends dependent on which anti-angiogenic agent is being studied, making their biomarker value deferential (Farace F *et al* 2007; Duda D *et al* 2008; Michael A *et al* 2010).

Table 5.1: List of agents shown to inhibit tumour angiogenesis

Angiogenesis Inhibitors
Tissue inhibitors of MMP (TIMP)
Thalidomide, Lenalidomide, Pomalidomide
Suramin and analogues
Fumagillin and TNP470 analogue
Cytokines
CAI
Endostatin
Angiostatin
Thrombospodin
Arginine Deiminase
Anginex
Anti-VEGF antibodies (Bevacizumab / Avastin)
Anti-VEGFR antibodies (DC101)
BAY 43-9006 (Sorafenib/ Nexavar)
SU5416 (Semaxanib)
SU6668
SU11248 (Sunitinib / Sutent)
PTK787/ZX222584 (Vanatanib)
ZD6474 (Vandetanib / Zactima)
GW786034 (Pazopanib / Votrient)
AZD2171 (Cediranib / Recentin)
Robo4
EGFR inhibitors
COX-2 inhibitors
mTOR inhibitors
Chemotherapy (metronomic) e.g. temozolomide

Table adapted from (Horsman M *et al* 2010)

5.1.1 Rationale for and potential applications of the enumeration of CECs and CEPs in clinical oncology

The role of angiogenesis in tumour growth has already been covered in depth (see chapter 1). The role of CECs and CEPs will be described in detail in this section, along with the rationale behind their enumeration in malignant melanoma.

Increases in number of circulating endothelial cells (CECs) and progenitors (CEPs) have been reported not only in association with malignancies, but also in various pathological conditions e.g inflammatory arthritis and macular degeneration (Kerbel R 2000). CEC and CEP kinetics have been shown in preclinical and clinical studies to correlate with angiogenesis assays and to be a potential predictive tool for clinical outcomes in patients undergoing anti-angiogenic treatment (Rugo H *et al* 2006; Farace F *et al* 2007; Duda D *et al* 2008).

CECs have a mature phenotype and are probably derived from blood vessel wall turnover (Lin Y *et al* 2000). This is a rare population, accounting for approximately 1 in 1000 circulating blood cells (or 0-20 CECs per mL) in the healthy individual (Blann AD *et al* 2005). It is a stable cell population, with the exception of a two-fold increase in women during the active menstrual cycle, associated with uterine remodelling (Mancusco P *et al* 2001). A subpopulation of CECs shows a progenitor-like phenotype. These have been shown to be able to incorporate into cancer vessel walls, although at low frequencies (Davidoff AM *et al* 2001). Some studies suggest that CEPs are paramount in promoting cancer vasculogenesis and are responsible for most endothelial cell proliferative potential (Ruzinova M *et al* 2003; Duda D *et al* 2006). However, as previously described in section 5.1, low cell numbers of CECs

and CEPs, render assays highly susceptible to errors in sampling, preparation and analysis (Strijbos M *et al* 2008). Several groups, for instance, have demonstrated the negative impact of venepuncture, as traumatically detached CECs contribute significantly to CEC counts (Goon P *et al* 2006; Rowland JL *et al* 2007). Because of the low numbers, enrichment steps are helpful, although inevitably lead to cell loss and underestimation of actual CEC/ CEP numbers.

Both these cell types pose as potentials for drug- targets to induce an anti-angiogenic response. **Figure 5.1** shows potential routes via which traditional chemotherapy drugs can achieve anti-angiogenic effects. As previously described, VEGF is a potent mobiliser of CECs and a pro-survival factor for differentiated, activated endothelial cells. Inhibition of VEGF or VEGFR has been shown to be clinically effective in treating renal cell carcinoma, which is a highly-vascular type of tumour (Board R *et al* 2007; Motzer RJ *et al* 2007; Escudier B *et al* 2008) .

The introduction of monoclonal antibodies (e.g.CD146) (Dignat-George and Sampol J 2000) with specificity for endothelial cells has led to the development of two main techniques for CECs and CEPs to be isolated: immunomagnetic bead selection and flow cytometry. Over the last two decades, immunomagnetic bead selection has continued to be developed (George F *et al* 1992; Dignat-George and Sampol J 2000; Woywodt A 2006). It initially involved the coupling of magnetic dynabeads to an anti-CD146 monoclonal antibody and then mixing them with peripheral blood. Unbound cells are washed out, but bound cells are magnetically retained. CECs are identified by the expression of von Willibrand factor (a blood glycoprotein involved in haemostasis) and the absence of CD45 (a transmembrane protein tyrosine phophatase present on all differentiated

Figure 5.1: Chemotherapy antiangiogenic targets

a)   targeting bone-marrow derived proangiogenic cells that adhere to the walls of new blood vessels and stimulate further growth via paracrine mechanisms
b)   targeting cycling endothelial cells present in sprouting capillaries of tumours
c)   bone-marrow derived CEPs that incorporate into the lumens of growing vessels and differentiate into endothelial cells.

Diagram adapted from (Ferrara N and Kerbel 2005).
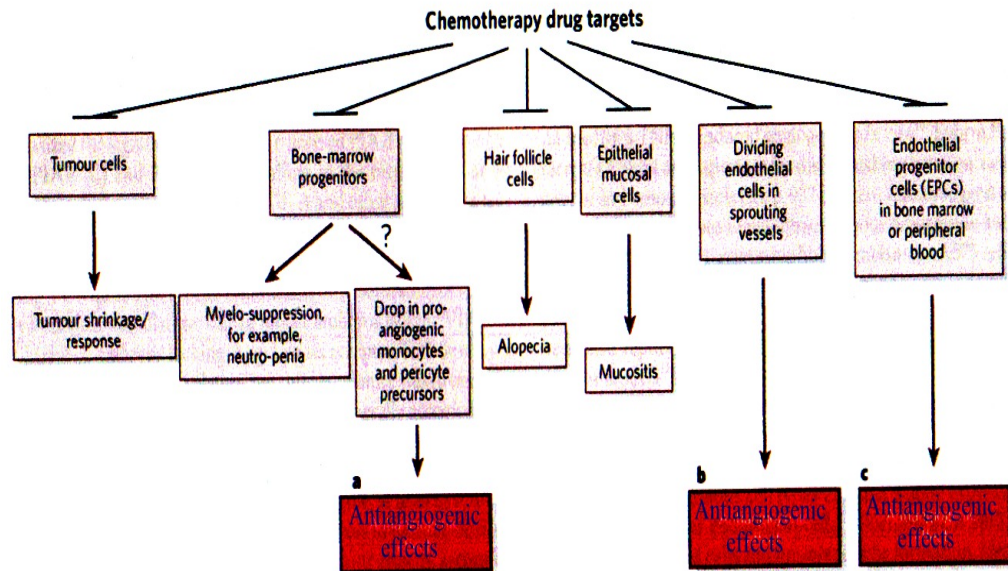
haematopoietic cells except erythrocytes and plasma cells) (Bertolini F *et al* 2006). This technique has been revised and refined, with multiple surface markers (eg CD146, CD31, CD34) being used to define CEC populations by multiparameter flow cytometry (Blann AD *et al* 2005; Strijbos M *et al* 2008).

There is however, a lack of consensus on a common endothelial cell phenotype. To date, the numerous studies investigating CECs and CEPs use a broad spectrum of cell markers e.g. CD31 (Beerepoot L *et al* 2004), CD34 (Furstenberger G *et al* 2006), VWF (Woywodt A *et al* 2006) for CECs and CD34 and CD133 (Yin A *et al* 1997) for CEPs. Published assay techniques define CECs as positive for CD146 (Mancusco P *et al* 2006). There is however, data to suggest that CD146 is expressed on vascular endothelium but not on viable CECs (Duda D *et al* 2006). There is also evidence that CD146 is also expressed on active lymphocytes, which are frequently increased in cancer patients, therefore skewing the results of studies using this as their cellular marker (Elshal M *et al* 2005; Duda D *et al* 2006). For these reasons, CD146 was not used as the cell marker in this study.

CEPs are present at a lower percentage in comparison to mature CECs and remain proliferative in nature. They are CD133 positive compared with mature CECs which are negative for this marker (Rafii S *et al* 2002), but a clear distinction between CEPs and haematopoietic stem cells (HSCs) is hampered by their expression of CD133, making this a current limitation in the sub-set isolation process. At the current time, the best way to distinguish between CEPs and HSCs is by their function rather than by their phenotype alone (Peters B *et al* 2005; Mancusco P *et al* 2006; Roodhart J *et al* 2010).

Preclinical studies have previously indicated that the counting of CECs and CEPs can be used to identify the optimal biological dose (OBD) in cancer-bearing mice treated with low-dose metronomic chemotherapy (Shaked Y *et al* 2005). Other studies have shown that, with antiangiogenic therapy, the number of these cells changes. Beaudry et al showed that with VEGF-inhibition in tumour bearing mice, the number of mature CECs increased (Beaudry P *et al* 2005). Further clinical studies have shown increased numbers of mature CECs in patients with progressive cancer, and increased apopototic CECs in patients on metronomic therapy. Enumeration of these cells has therefore been shown to have a potential role in therapeutic and monitoring strategies, particularly in anti-angiogenic cancer treatment (Kerbel RS and Kamen 2004; Dellapasqua S *et al* 2008; Roodhart J *et al* 2010).

The key differences between MTD regimens and metronomic therapy have been described in the introduction to this thesis (see section 1.3.1). In contrast to MTD chemotherapy, metronomic chemotherapy regimens maintain the low levels of viable CECs for longer periods of time due to the absence of break periods (associated with surges in tumour neoangiogenesis) (Carmeliet 2003). The addition of an anti-angiogenic agent to the traditional MTD regimens has been shown to prevent the rebound mobilisation of CEPs after MTD therapy (Browder T *et al* 2000; Bertolini F *et al* 2003; Kerbel RS and Kamen 2004; Schenider M *et al* 2005). Bertolini and colleagues showed in a preclinical study that metronomic chemotherapy was associated with a consistent decrease in CEP numbers and viability and a more durable inhibition of tumour growth although the clinical significance of this remains uncertain. (Bertolini F *et al* 2003). Beaudry's preclinical study indicated that anti-

angiogenic drugs might have dual effects on CECs and CEPs, such as an increase in CEC count that parallels the inhibition of CEP mobilisation in the peripheral blood (Beaudry P *et al* 2005).

CEC and CEP enumeration correlate well with preclinical gold standard angiogenesis assays and hold potential for OBD determination and disease monitoring. The application of CEPs as a potential vehicle for anticancer treatments has also been studied (Ferrari N *et al* 2003; Dome B *et al* 2006). Provided that an adequately validated, sensitive and specific assay is used, the detection and enumeration of CECs and CEPs in patients with solid malignancies offers a wide spectrum of applications. Some of these are detailed below:

a) Prognostic marker

It has been observed that some cancer patients, including breast, hepatocellular, renal cell and colorectal cancers, show higher CEC counts than healthy controls, whereas patients with stable disease have lower numbers, equivalent to disease-free individuals (Duda D *et al* 2008; Rugo H *et al* 2005; Malka D *et al* 2007, Farace F *et al* 2007). This area requires further study in large randomised controlled trials that include anti-angiogenic regimens. As the field progresses, individual differences need to be taken into consideration, but CECs may well serve as a common indicator of disease relapse .

b) Marker for therapy-induced response

Radiological assessments to look at tumour size as a marker of antitumour effects of therapy are now thought to be insufficient, despite recent advances in RECIST (Therasse *et al* P 2009). This is particularly true in the case of anti-angiogenic regimens, where enhanced progression-free periods have been observed, yet response

rates as defined by conventional imaging, were minimal (Yang J *et al* 2003). More sensitive indicators of response are therefore needed. Diminished CEC levels have been shown in mice treated with endostatin, an angiogenesis inhibitor (Schuch G *et al* 2003). CEC and CEP kinetics and viability have also shown to predict clinical response in some metronomic regimens (Dome B *et al* 2006; Mancusco P *et al* 2006; Dellapasqua S *et al* 2008; Roodhart J *et al* 2010).

c) Guideline for optimal drug dosing

The OBD is the dose that is feasible to be safely applied to humans and likely to yield biological effects. This dosing method supports the metronomic theory as described in chapters 3 and 4 (Bertolini F *et al* 2003; Maraveyas A *et al* 2005; Lam T *et al* 2006).. Thus the concept of MTD will need to be redefined as a dose that can be administered safely over a long duration . Changes in CEC and CEP counts after dose escalation might provide useful insights in establishing the OBD when assessing anti-angiogenic agents. One study showed that the OBD, determined by assessment of tumour microvessel density and analysis of tumour blood flow, resulted in the largest decrease in CEC numbers. Also, administration of varying amounts of endostatin (a 'broad-spectrum' antiangiogenic) resulted in a clear 'U-shaped' dose-response curve on CEC percentage (Celik I *et al* 2005). See **Figure 5.2.** Clinical studies have since shown the prevention of CEP and CEC mobilisation when using the OBD or metronomic approach to dosing (Stoelting S *et al* 2008).

d) Marker for vascular toxicity

With the suggestion from some studies that the incidence of cardiovascular events has now emerged as one of the most important sequelae of long-term anti-cancer therapy, the use of CEC and CEP measurements to predict this has been suggested. *In vitro*

Figure 5.2: Inhibition of CECs by Endostatin – the 'U-shaped curve'

This study was carried out *in vivo* (mouse-model) and clearly shows that there is a range of dosing of Endostatin where CEC mobilisation / viability is kept to a minimum, but that too high a dose can actually induce CEC stimulation / mobilisation. This is a biphasic dose-response curve, not a linear one. It is similarly shown by IFN-α as reported by Slaton et al 1999.
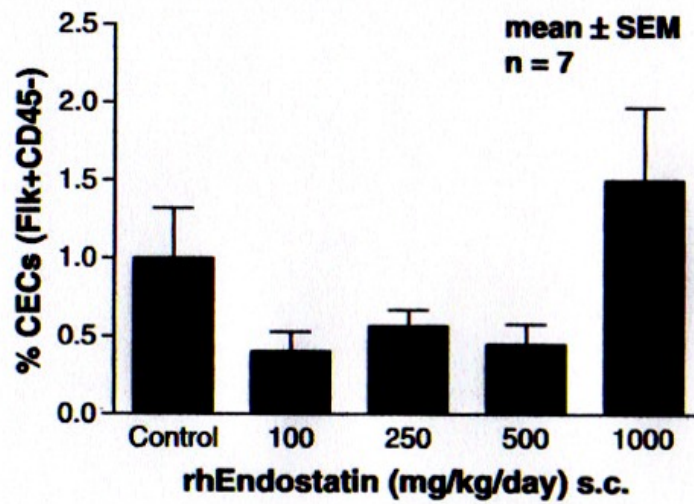
Diagram taken from (Celik I *et al* 2005).

and clinical evidence suggests that cytotoxic agents can directly damage endothelium (van Heeckeren W *et al* 2006). Commonly used agents such as cisplatin, etoposide and bleomycin can cause thickened arteries, Raynaud's syndrome, increased VWF and plasma C-reactive protein, associated with endothelial cell dysfunction (Nuver J *et al* 2005). Monitoring CECs throughout treatment might give a closer insight into the vascular toxicity profile of chemotherapeutic agents. This data could be used to select less vasotoxic treatments, especially for those patients likely to require long-term therapy e.g. germ-cell cancers, lymphoma or for those with coexisting cardiovascular morbidity.

Figure 5.3 shows the hypothesis of CEC kinetics with treatment. It is thought that with the onset of anti-angiogenic therapy, mature CECs initially increase (mainly apoptotic CECs) where as CEPs decrease. As tumour load reduces, CECs are also reduced. Relapse or failure of response to treatment may be marked by an increase in both of these cell populations .

With anti-angiogenic therapy being currently one of the key areas of interest in modern clinical oncology, particularly in the multi-targeted regimens, such biomarkers are essential, particularly in solid malignancies where new treatment strategies are urgently needed. This is certainly the case for malignant melanoma.

5.1.2 Rationale behind comparing conventional-dose chemotherapy to metronomically-dosed chemotherapy in melanoma

Advanced melanoma remains uniformly fatal and systemic therapy induces durable

Figure 5.3: Hypothesis of CECs kinetics with treatment

Hypothesis of CECs kinetics with treatment
(A) predicted change in mature CECs and CEPs that could accompany antiangiogenic treatment. On treatment initiation, CECs tend to increase and CEPs decrease. As tumour burden decreases, so does the mature CEC count. It is possible that tumour relapse may be indicated by a surge in both cell types. The initial CEC surge is accounted for by a surge in CEC apoptosis (B).
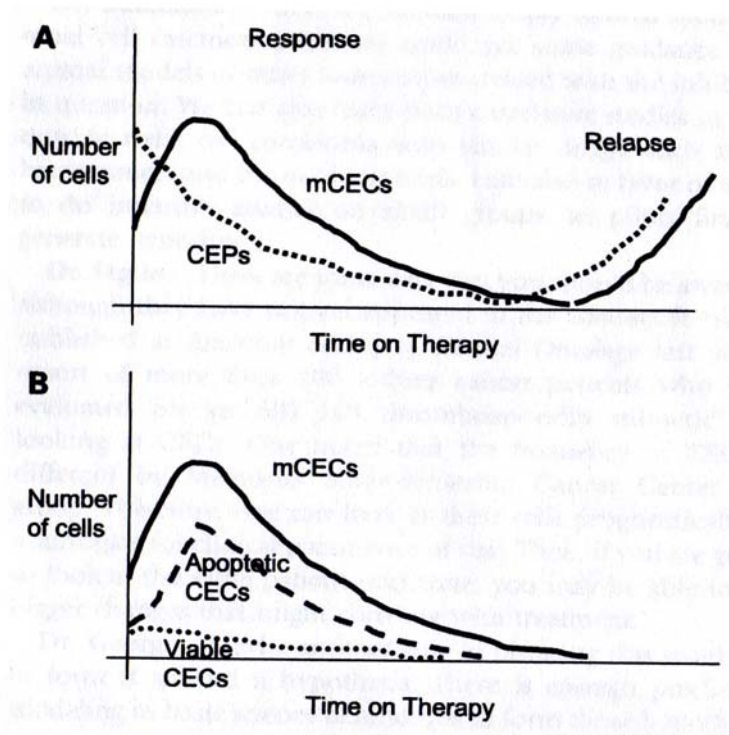
Diagram taken from (Bhatt R *et al* 2007).

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responses in a small minority of patients. The introductory section on the oncologists challenge in treating melanoma describes this in more detail (section 1.4.5). There remains an urgency to find better regimens and to compare their efficacy with older conventional regimens. Dacarbazine (DTIC) has historically been the first line single-agent chemotherapy drug for advanced melanoma. Its reported response rates of 20% have been dispelled by randomised controlled trials that confirm response rates of only 7.2-7.5% (Avril MF *et al* 2004; Bedikian A *et al* 2006). Despite this, DTIC remains the only cytotoxic drug approved by the U.S Food and Drug administration for the treatment of metastatic melanoma, and is still the mainstay of many combination regimens (see section 1.4.5 and 3.1.2.1).

Temozolomide, as discussed in section 3.1.2.1, has shown equivalent efficacy to DTIC in terms of objective response rate, time to progression and overall disease free survival (Middleton M *et al* 2000). So far, only a moderate difference in PFS compared to a standard dosing regimen when in combination with sorafenib has been shown in melanoma patients, as discussed in section 4.3 (Amaravadi R *et al* 2009).

The lack of viable alternatives has often driven the conduct of combination trials in melanoma rather than true biologic rationale based on laboratory or clinical evidence of synergistic activity. Modest phase II results have led to disappointing phase III results. These have been mainly using sorafenib and bevacizumab, in combination with DTIC, Temozolomide and other cytotoxics (Amaravadi RK *et al* 2007; Escudier B *et al* 2008; McDermott D *et al* 2008; Amaravadi R *et al* 2009; Hauschild A *et al* 2009). As previously discussed, such failures may be attributed to empiricism of trials rather than because of proof of synergy or mechanism of action through tissue studies

(Maraveyas A *et al* 2005; Gogas H *et al* 2007). Pre-clinical work is therefore essential in this confusing era of multi-targeted combination regimens.

This pilot study therefore aims to enumerate CECs and CEPs in patients with metastatic melanoma receiving metronomically-dosed chemotherapy. This patient group was compared to those receiving conventional chemotherapy based on MTD, and also to healthy controls with the objective to see whether we could identify any difference in the kinetics of these circulating markers. The treatment protocol devised for this study was based on a dose escalation schema to achieve the metronomic or OBD (Maraveyas A *et al* 2005; Lam T *et al* 2007) (see section 2.2.1, table 2.1.). Enumeration of CECs and CEPs in the chosen patient groups before, during and after treatment was carried out, aiming to obtain proof of principle data.

This chapter focuses on the clinical arm of the study of anti-endothelial treatment in patients with stage IV metastatic malignant melanoma. The patient group is described in more detail in **Table 5.2**, but in summary they fall into 2 groups - one receiving conventional MTD chemotherapy (DTIC plus vindesine) and the other receiving extended-schedule oral temozolomide and estramustine (see table 2.2). The metronomic group were patients recruited into the study at level 5a and 6 (see table 2.2) of the dose-escalation scheme of the the phase I/II study of temozolomide and estramustine. At these dosing levels, (described in section 2.2.1) no conventional bone marrow toxicity (as monitored by serial full blood count measurements) had been seen and 33% of MTD was not exceeded, as per metronomic principles.

Table 5.2: Summary table of study participants and their demographics

*Conventional – DTIC+Vindesine; *Metronomic – Temozolomide+Estramustine (at dose level 5a/6)

Patient	Age at presentatio	Sex	Primary site & Breslow thickness	Site of metastatic disease	Stage/TNM start of study
Conventional MTD*DTIC-Vindesine*					
1	48	M	R chest – 4.7mm	Axillary (1/20 nodes), pulmonary+mediastinal	IV T4N1M1b
2	63	M	Back – 1.9mm	Axillary (1/20 nodes), pulmonary	IV T2bN1aM1b
3	35	F	L heel – 2.35mm	Groin (5/15 nodes), pulmonary, mediastinal, local recurrences	IV T3aN3M1b
4	58	F	R loin – inconclusive Breslow but S100+	R inferior pubic ramus (associate pathological fracture). Refer to case report Appendix 4.	IV T?N0M1c
Metronomic 'Level 5a and 6 Temozolomide –estramustine-*					
1	70	M	R flank – 5 mm	Hepatic + pulmonary	IV T4N0M1c
2	52	F	Posterior neck -0.52 mm, Right abdomen – 3.1 mm	Axillary, multiple pulmonary	IV T3N1aM1b
3	39	F	L foot – 5.4 mm	L groin, hepatic	IV T4N2aM1c
4	67	M	L inner canthus eye – 2.3mm	L neck nodes, hepatic, local recurrence	IV T2N1bM1c

5.2 Optimisation methodology and control samples

The initial stages of CEC and CEP isolation involved a series of optimisation experiments. For this, blood samples were first taken from an additional 4 healthy volunteers – not those used as the study control group (**table 5.3**). These volunteers were seen at the 'benign skin lesion clinic'. They were all given the relevant information sheet and consent form (LREC number 06/Q1105/38; HEY number R0397; see Appendix 2.3 and 2.4) prior to participating. Additional samples from 3 patients outside the study group with known stage III or IV metastatic melanoma were also used in the optimisation process. These were surplus samples from a previous similar study carried out within the department studying the impact of Interferon alpha on CECs and CEPs. Ethical approval had already been gained for this previous study (LREC number 05/Q1105/76) and therefore all participants were fully informed and consented. Blood samples had been collected and stored using the same methodology as used in this study.

5.2.1 The freeze-thaw process optimisation

The freeze-thaw process (see section 2.2.2.4) was initially optimised to ensure frozen vials had a high yield of live cells. Yield was improved by using all reagents at $4^{\circ}C$ throughout, by increasing centrifugal force to 1500rpm and by increasing washing steps from one to three to minimise debris. These steps ensured that cell yield averaged around 1×10^{7} per vial. There was a moderately better cell yield (1.5-1.7×10^{7}) seen when working with fresh blood directly through all steps rather than with frozen samples. However, given the infrequency of patients entering this particular study, the random times of their clinic attendances – often changed at short

notice, the high cost of reagents and the short expiry dates on microbeads and antibodies, this was deemed an impractical way to work. The optimised freeze-thaw process was therefore used, and samples processed in batches.

Table 5.3: Healthy volunteer demographics

Healthy volunteer	Age	Sex
1	67	M
2	32	F
3	50	M
4	29	F

5.2.2 Microbead-cell adherence

The next stage of optimisation involved ensuring that the endothelial cells were actually adhering to the specific microbeads. This was confirmed by microscopic examination and by using a magnet adjacent to the microscope slide. Visible movement was seen under microscopic examination of the cell-bead duo towards the magnet when this manoeuvre was carried out, providing evidence for the adherence of the microbeads to endothelial cell walls.

5.2.3 Optimising selection

The depletion and positive selection stages were looked at, to ensure that CECs and CEPs were not being lost in the process. From a control sample (healthy volunteer) of 3×10^7 cells/mL, cell counts showed 1.7×10^7 cells/mL in the CD45 positive fraction and 1.6×10^6 cells/mL in the CD45 negative fraction. The CD45 positive fraction was

then incubated with CD34-FITC and CD133-PE to see if any of the wanted cells (CECs and CEPs) were retained in the column. The CD34 negative fraction collected after positive selection for CD34 was also incubated with CD34-FITC and CD133-PE to see if any of the wanted cells had passed through the column, either due to bead-saturation or for other reasons.

Next, the CD45 depleted sample chosen for use was checked for the presence of stray CD45 positive cells using CD45 microbeads and CD45 FITC to ensure that the initial depletion process had been satisfactory and that the results were not skewed by a high proportion of CD45 positive cells. This showed that 0.01% of the CD45 positive fraction contained CD34 positive cells and that 0.06% of the CD45 negative fraction contained CD45 positive cells.

5.3 Results

The aim of this scientific 'pilot' sub-study was to compare the numbers of CECs and CEPs (measured in cells/µL of whole blood) between the conventionally-dosed and the metronomically-dosed patient groups at different stages in their treatment cycles. A control group of age and sex matched healthy volunteers was also used for comparative purposes.

Results for the 'conventionally-dosed' and 'metronomically-dosed' patient groups are as shown, with healthy volunteer results listed below. Results are displayed below in **table 5.4.**

Table 5.4: Results of CEC/CEP enumeration in the study groups

Patient	CEC/CEP baseline (cells per µL) – Week 0	CEC/CEP mid-treatment (cells per µL) – Week 6	CEC/CEP post-treatment (conventional) or at 3 months (metronomic) (cells per µL) – Week 12
Conventional			
1	8.14 / 0.68	5.43 / 0.59	11.53 / 0.88
2	13.27 / 0.23	9.62 / 0.21	15.27 / 0.51
3	17.16 / 0.19	12.83 / 0.13	18.34 / 0.46
4	17.45 / 0.31	10.33/ 0.26	2.32 / 0.38
Metronomic			
1	8.00 / 0.03	9.00 / 1.65	11.67 / 0.95
2	4.27 / 0.12	13.81 / 0.1	15.26 / 0.12
3	3.03 / 0.09	9.32 / 0.12	9.06 / 0.08
4	10.92 / 0.62	12.88 / 0.61	8.54 / 0.54
Healthy Controls			
1	1.01 / 0.0		
2	1.12 / 0.0		
3	0.45 / 0.02		
4	0.86 / 0.0		

*results are based on mean of duplicates and expressed in cells / µL of whole blood.

As can be seen from the results, there were 4 participants in each study group. CEC and CEP were estimated in both the control and treatment groups to obtain baseline (pre-treatment) data. The metronomic group were all chemo naïve for the pre-treatment sample. Repeated measurements of CEC and CEP were made from the two treatment groups mid-treatment (6 week cohort) and post-treatment (12 week cohort) – see table 2.2 for dosing schedule.

5.3.1 Analysis of results

Kolmogorov-Smirnov tests indicated that the distributions of the estimates of CEC/µl and CEP/µl did not deviate from normality justifying the use of parametric statistics. There was significant variability between all groups in both CEC and CEP numbers, reflected by wide confidence intervals. Detailed statistical results can be seen in **appendix 3**.

The mean CEC (at baseline) was lower in the control (healthy) group (0.86±0.3) than in the conventional and metronomic treatment groups (14.01±4.3 and 6.55±3.6 respectively). There was no significant difference between the mean CEP estimates in the control and treatment groups (p=0.103) (see **figure 5.4 and 5.5**).

The mean difference between the pre-treatment minus the mid-treatment CEC estimates for the conventional group was positive, indicating that the mid-treatment CEC was lower than the pre-treatment CEC (see **figure 5.6**). The mean difference between the pre-treatment minus the mid-treatment CEC estimates for the metronomic group was negative, indicating that the mid-treatment CEC was higher than the pre-treatment CEC.

Figure 5.4: Comparison of the baseline (pre-treatment) mean CEC/µl ± 95% confidence intervals in the three groups (n=4 each group).
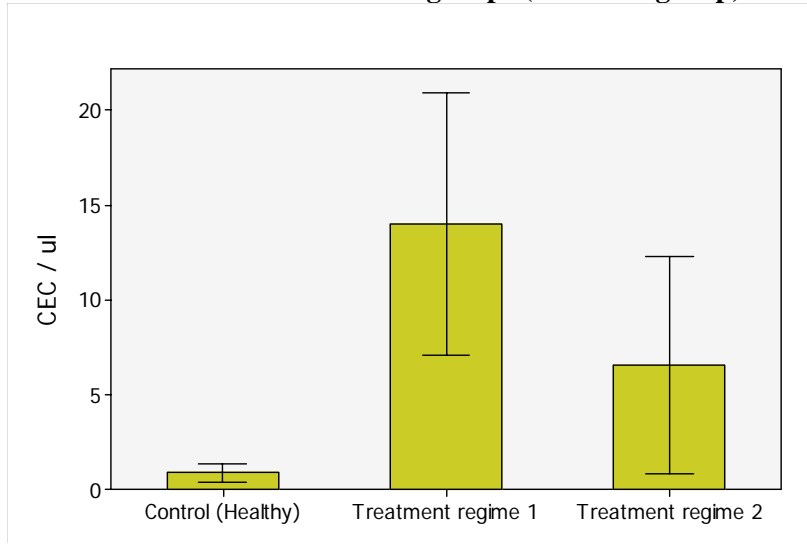
Figure 5.5: Comparison of the baseline (pre-treatment) mean CEP /µl ± 95% confidence intervals in the three groups (n=4 each group).
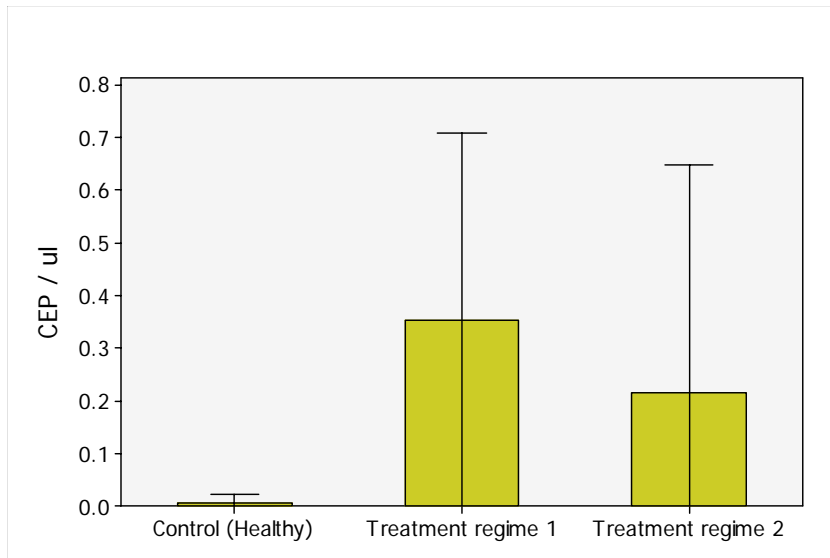
Regime 1: Conventional

Regime 2: Metronomic

Figure 5.6: Comparison of the differences (pre-treatment minus mid-treatment) between the mean CEC/µl ± 95% confidence intervals in two groups of cancer patients (n=4 each group).
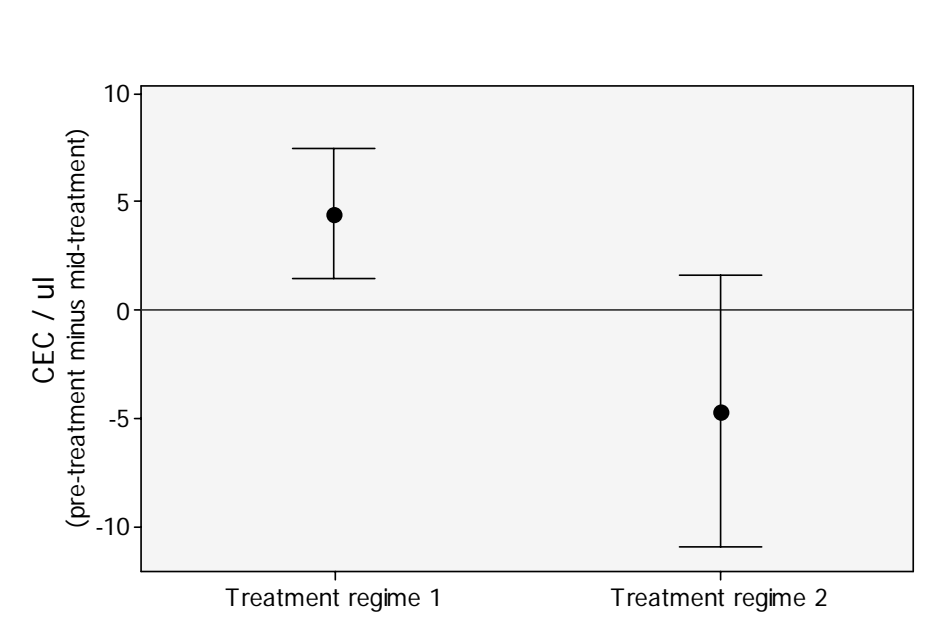
Acquired histogram examples of patient 4 from the conventional group and patient 1 from the metronomic group demonstrate this finding (see **figure 5.7a and b**).

The mean difference between the pre-treatment minus the post-treatment CEC estimates for the conventional group was positive, indicating that the post-treatment CEC was lower than the pre-treatment CEC (see **figure 5.8**). The mean difference between the pre-treatment minus the post-treatment CEC estimates for the metronomic group was negative, indicating that the post-treatment CEC was higher than the pre-treatment CEC.

The mean difference between the pre-treatment minus the mid-treatment CEP in the

Figure 5.7: FACS-generated histograms: patient results

a) Patient 4 on conventional treatment: a reduction in mid-treatment CEC levels is seen. This was the only patient in the conventional group to remain disease free at 3 years

Pre-treatment CEC followed by mid-treatment CEC (negative control shown as green peak to the left).
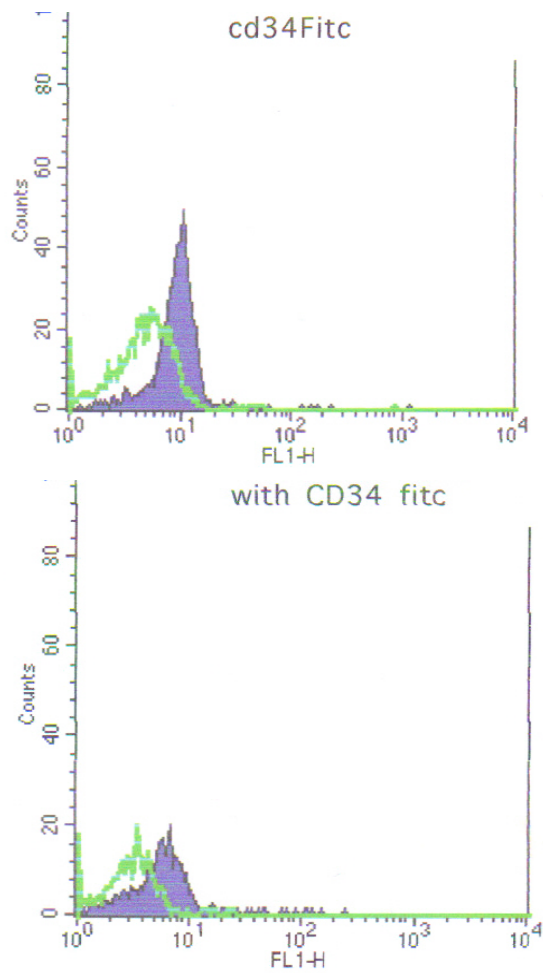
Figure 5.7:

b) Patient 1 on metronomic treatment: a minimal reduction in mid-treatment CEC levels is seen.

Pre-treatment CEC followed by mid-treatment CEC
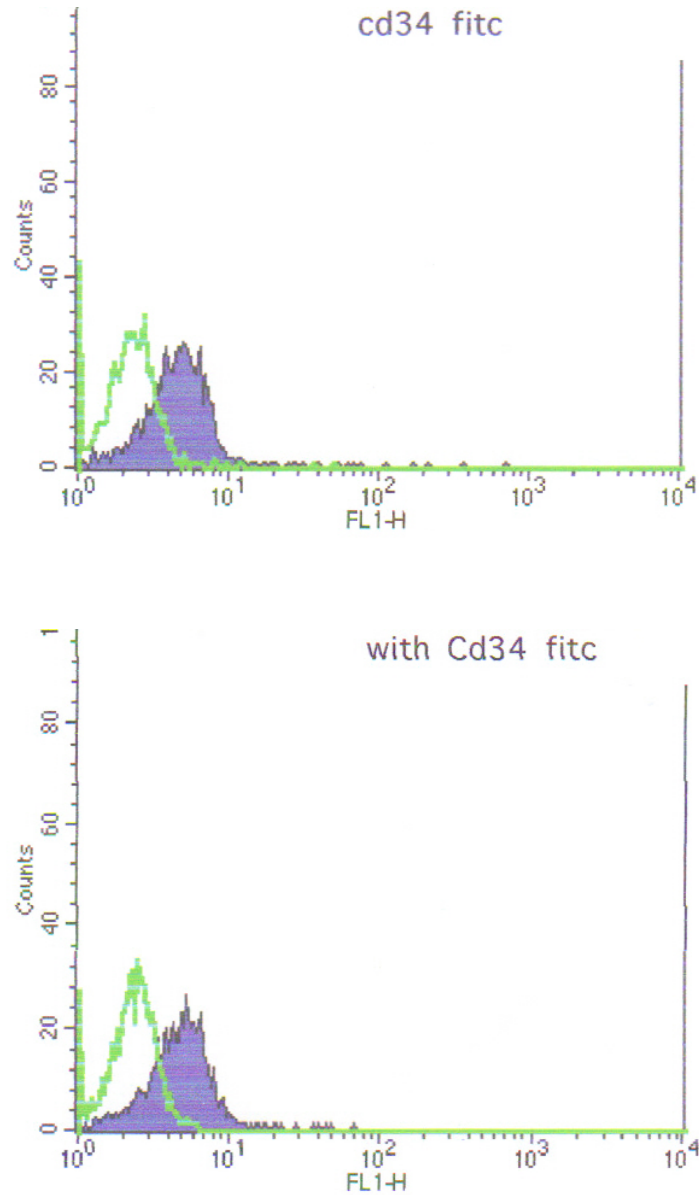
Negative control shown as green peak to the left

Figure 5.8: Comparison of the differences (pre-treatment minus post-treatment) between the mean CEC/µl ± 95% confidence intervals in the two groups of cancer patients (n=4 each group).
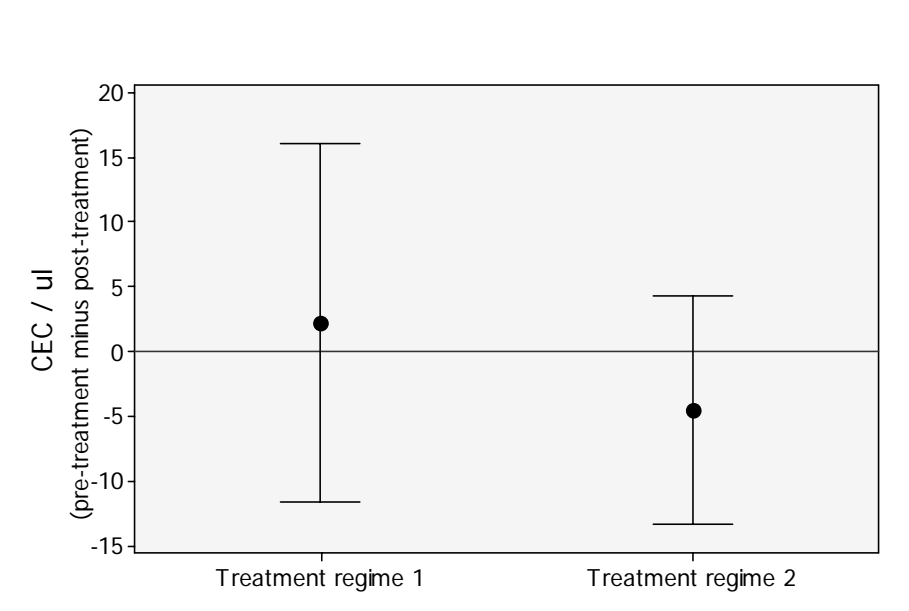
conventional group was positive indicating that the mid-treatment was lower than the pre-treatment, however in the metronomic group, this was negative, indicating that the mid-treatment was higher than the pre-treatment (see **figure 5.9**).

The mean difference between the pre-treatment minus the post-treatment CEP in the conventional group was negative, indicating that the post-treatment CEP was higher than the pre-treatment. This was also the case for the metronomic group, indicating that the post-treatment CEP was also higher than the pre-treatment (see **figure 5.10**).

Accepting that the study is a very small pilot the following tentative conclusions can be drawn from the statistical analysis of these results:

• The mid-treatment CEC was significantly lower than the pre-treatment CEC in the conventional group, whereas it was significantly higher than the pre-treatment CEC in the metronomic group. The effect size was substantive (see **figure 5.7** for example histograms).

• There were no significant differences between the pre-treatment mean CEC and the post-treatment mean CEC in the two groups of patients. Similarly, there were no significant differences between the pre-treatment mean CEP and the mid-treatment mean CEP or the post-treatment mean CEP in the two groups. The effect sizes were relatively small.

• There was a general trend for CEC to be relatively more variable than CEP, and for the effect sizes of CEC to be larger than CEP, with respect to both time and treatment.

Figure 5.9: Comparison of the differences (pre-treatment minus mid-treatment) between the mean CEP/µl ± 95% confidence intervals in two groups of cancer patients (n=4 each group).
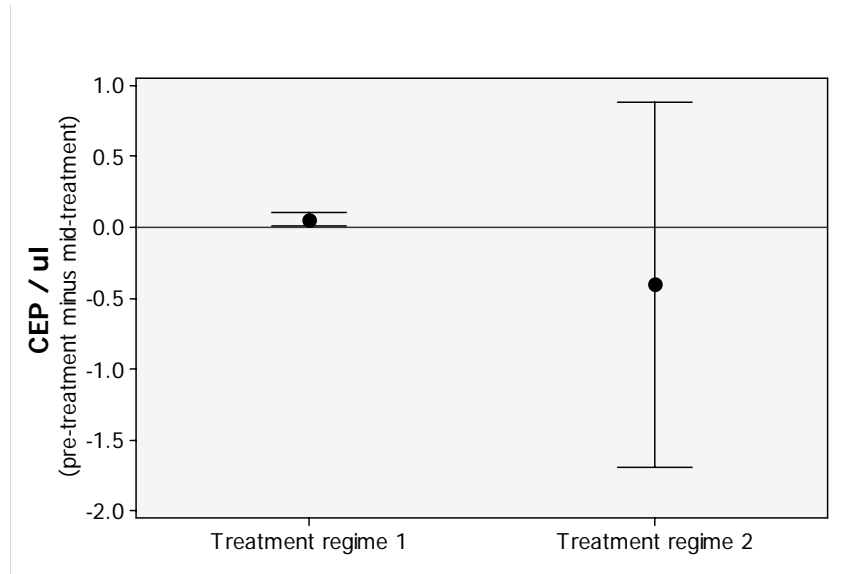
Figure 5.10: Comparison of the differences (pre-treatment minus post-treatment) between CEP/µL ± 95% confidence intervals in two groups of cancer patients (n=4 each group).
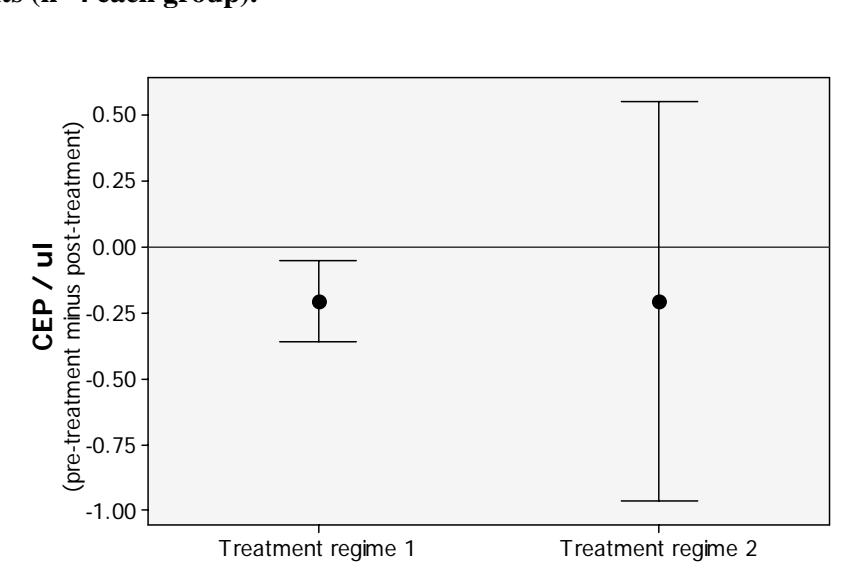
Regime 1: Conventional
Regime 2: Metronomic

Patient 4 from the conventional group showed a different pattern of CEC levels throughout treatment to the other patients in this group. This patient had a further fall in CEC levels at week 12. Interestingly, this patient is the only one from the group that had a partial response to treatment and remained disease free at 3 years. She did however develop metastatic end-stage disease subsequent to this period (see **appendix 4** for case report). **Figure 5.7** demonstrates this patient's mid-treatment fall In CECs.

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5.4 Discussion

This study has involved setting up and optimising a methodology for isolating and enumerating CEPs and CECs, as set out in the study aims. In terms of the methodology, several groups have previously demonstrated the negative impact of venepuncture (Goon P *et al* 2006; Rowland JL *et al* 2007). In addition, the whole blood samples may have produced a better cell yield had the work not required the use of the freeze-thaw process. Because of low cell numbers, enrichment steps were used, but these steps can also lead to cell loss. This was considered and cell loss was tested in the optimisation experiments.

This is a very small scientific study with many limitations, but designed as a hypothesis-generating study. A significantly larger sample of stage IV melanoma patients would be required in order to test the usefulness of this tool as a potential biomarker. This could again be a sub-study of a much larger randomised study where clinical end-points can be correlated to the changes seen. This would also require the metronomic group to be on an equivalent oral version of the conventional group agents. In this case, our two treatment groups are on different agents, although the

DTIC and temozolomide are very similar agents.

Previous studies have shown great variabilities in cell counts, dependent on variability in sample processing, antibody used, setting of gates and differences in equipment and FACS machines. In order to compare results between studies, it would be necessary to have a uniform approach, or for an assay to be developed that could be reproducible and precise. In the interim, comparing trends may be more useful, and seeing if CEC and CEP levels truly correlate with a clinical anti-angiogenic response to treatment. At the moment, our main tools for reponse to anti-angiogenic treatments include microvessel density (as discussed in section 1.2), vascular-specific radiological screening (e.g. dynamic CT and PET scans), and blood tests such as serum VEGF and VEGFR2 measurements. The lack of a 'gold standard' VEGF detection test is a significant hindrance to the clinical utility of VEGF measurements. Along with this, VEGF levels also fluctuate with temperature and clotting duration, making it currently an unreliable biomarker (Fuhrmann-Benzakein E *et al* 2000; Jubb A *et al* 2006).

This small study is however, a potentially useful starting point for further work. As previously discussed, it is imperative that reliable assays for biomarkers are developed to be used in monitoring patients with malignant melanoma and other cancers. This work should only be seen a generating some more hypotheses and providing some intriguing early 'proof of principle' data only. It is the first study to focus on CEC and CEP enumeration in the context of metronomic versus conventional therapy in malignant melanoma. Other studies quantifying these cells have tended to look at them in the context of metronomic, anti-angiogenic regimens

e.g low-dose cyclophosphamide, methotrexate +/- thalidomide (Mancusco P *et al* 2006).

When looking at previous related work, Mancoso *et al* detected increased amounts of CECs in cancer patients using flow cytometry (Mancusco P 2001). CECs numbers were shown to decrease in patients with lymphoma after complete remission following chemotherapy, and also after mastectomy in breast cancer patients. Mancusco's work also showed that patients with progressive cancer of varying types – including 3 patients with melanoma - have more CECs than age and sex matched healthy volunteers (95 patients, 438 ± 65 CEC/ml; P<0.001 by Mann-Whitney test). This finding was also seen in the results of this study, with healthy controls having a mean CEC value of 0.86 CEC/µL, and patients with melanoma having pre-treatment mean CEC value of 10.28 CEC/µL. Another finding from previous work was that patients with stable disease showed comparable amounts of CECs to healthy volunteers (17 patients, 179 ± 61 CEC/ml; P=0.69 by Mann-Whitney test) (Beerepoot L *et al* 2004; Rowland JL *et al* 2007). This comparison could only be made with patient 4 (conventional treatment), since this was the only patient that had stable disease (for 3 years) post-treatment. This patient did however, have lower CEC values post-treatment (2.32 CEC/µL) although, unfortunately in the final stages of this thesis write-up, developed metastatic disease (see appendix 4 for further detail).

Other studies have looked at metastatic breast cancer patients, and have suggested that CEC levels are increasingly thought to reflect response to anti-angiogenic treatment, and that an increase at week 3 whilst on an anti-angiogenic regimen (including bevacizumab) predicted a worse PFS (0.015) (Rugo H *et al* 2005; Rugo H

et al 2006). Along with breast cancer, the predictive value of CECs / CEPs have also been assessed in the context of hepatocellular carcinoma, renal cell carcinoma, colorectal cancer and pancreatic cancer, with varying results (Iyer R *et al* 2006; Mancusco P *et al* 2006; Farace F *et al* 2007; Malka D *et al* 2007; Bidard F *et al* 2010; Matsusaka S *et al* 2010). For example, Mancusco *et al* showed that the CEC count after 2 months of continuous therapy (low dose cyclophosphamide and methotrexate) was a good predictor of disease-free and overall survival. In this study, patients with CEC $>11\mu g^{-1}$ blood after 2 months of this regimen, had a significantly improved survival. Another recent study showed that CEC count was associated with improved time-to-progession, at a threshold of 20 CEC/4mL ($p<0.01$). This was in metastatic breast cancer (n=67) in patients being treated with bevacizumab and chemotherapy (Bidard F *et al* 2010).

A number of studies have also shown a general trend (Bertolini F *et al* 2003; Furstenberger G *et al* 2006) to suggest that conventional chemotherapy evokes both an acute and a late systemic host response composed of release of CECs, CEPs and other factors. In the study by Furstenberger *et al*, MTD chemotherapy was shown to dramatically decrease CECs, where as pro-angiogenic CEP mobilisation was increased in drug-free periods. The extent of this CEC and CEP release seems to correlate with reponse to treatment and prognosis (Roodhart J *et al* 2010).

Another observation from this study was that the mid-treatment CEC level was significantly lower than the pre-treatment level in the conventional group, whereas the mid-treatment CEC level was significantly higher than the pre-treatment level in the metronomic group. A hypothetical model for CEC kinetics (see **figure 5.3**) was

proposed by Bhatt *et al* (Bhatt R *et al* 2007), and is consistent with clinical data (Mancusco P 2006) with a large initial large surge in CEC apoptosis secondary to anti-angiogenic treatment. Similarly, Celik *et al* demonstrated a dose-response relationship between endostatin, an anti-angiogenic, and CEC mobilisation (Celik I 2005). See **figure 5.2**.

Given the limitations of this study in terms of its very small size and minimal cell verification with additional markers (as discussed earlier in this chapter), it is not possible to say whether there are valid, clinically-relevant trends in the data. It is also not possible to draw conclusions from the results in terms of clinical outcome. Given the care taken to optimise the technique, it is hoped that the data is valid within the context of this study, and within the context of metastatic melanoma, in which biomarkers and new treatment strategies are urgently needed.

As discussed in the introduction to this chapter, there are numerous technical issues related to the methodology of CEC/CEP isolation. Cell numbers of CECs and CEPs are already low in peripheral blood. There was a significant possibility that cell numbers were reduced by the freeze-thaw process of sample preparation, and during the enrichment steps. Optimisation experiments did however attempt to detect and minimise cell loss by means of the enrichment steps by analysing samples that were to be discarded to check for the presence of CECs/CEPs (see section 5.2). A major disadvantage of this technique is that it is labour-intensive, requiring precise multiple steps, making it unsuitable for high throughput monitoring.

The lack of consensus on a common endothelial cell phenotype, as discussed in the

introduction, also poses problems for inter-study comparisons and validity of data. The methodology used in this study allowed specific cellular isolation of CECs and CEPs from whole blood. The methodology, however, lacks the detailed phenotyping of cells with additional markers for full verification (i.e CD146, CD31 or VEGFR2) (Furstenberger G *et al* 2006; Mancusco P *et al* 2006). For enumeration of the total number of CECs, and not a particular subpopulation, it is necessary to identify markers that are specific for and constantly expressed on CECs. See **table 5.5** for markers used in CEC assays in previous studies, showing the diversity of markers and their level of expression. No marker to date fulfils these necessary criteria completely, and as such, results between different studies are not comparable. It seems that only a multiparametric, concurrent investigation using a multitude of relevant antibodies can fully differentiate between CECs, CEPs and haematopoietic cells (Bertolini F *et al* 2006). For the purposes of a small study such as this, the practicalities of using multiple antibodies without larger sample numbers, could not be justified, although may have added weight to the findings.

CD133 is the sole antigen discovered to date, that is expressed in CEPs and subsequently downregulated in mature CECs (Rafii S *et al* 2002; Mizrak D *et al* 2008). In this study, the CEP measurements were unremarkable, with no particular observational variances. CEPs were defined as $CD45^-CD34^+CD133^+$. They too were likely diminished by the freeze-thaw and enrichment steps. Other studies have suggested that CEP levels appear to increase in response to anti-angiogenic agents (Beaudry P *et al* 2005; Farace F *et al* 2007). In this study, the effect of a molecularly-targeted antiangiogenic agent was not being assessed, and it may be that when such an agent e.g sorafenib, was added into the treatment regimen, CEP levels become more relevant as a biomarker.

Table 5.5: Markers used in CEC assays

Marker	Description	Subtype association	Expression level	Coexpression	References
CD31	PECAM-1	Panendothelial	++[a]	P, panleukocyte	(Beerepoot L 2004)
CD34	Stem cell marker	Panendothelial	++[a]	S	(Furstenberger G 2006)
CD36	Collagen receptor 1	Microvascular	+[a]	P,E,M,D	(Moroni G 2005)
CD54	ICAM-1	Inflammation	+[a]	L,M	(Dixon G 2004)
CD62-E	E-selectin	Inflammation	++	_	(Dixon G 2004)
CD62-P	P-selectin	Inflammation	+	P	(Corcoran T 2006)
CD105	Endoglin	Angiogenesis, malignant	++[a]	S,M[b]	(Rowland JL 2007)
CD106	VCAM-1	Inflammation, malignant	+[a]	_	(Dixon G 2004)
CD137	ILA/4	Malignant	+	L[b],D	(Seaman S 2007)
CD144	VE-Cadherin	Panendothelial	+[a]	_	(Smirnov D 2006)
CD146	MelCAM	Panendothelial	++[a]	L[b]	(Dignat-George F 2007)
CD202b	Tie-2	Angiogenesis	(+)[a]	_	(Smirnov D 2006)
CD276	B7-H3	Malignant	+	D,M[b]	(Seaman S 2007)
CD309	VEGFR-2	Angiogenesis	(+)[a]	S	(Beerepoot L 2004)
V-WF		Panendothelial	++[a]	P	(Woywodt A 2006)
UEA-1		Panendothelial	++	-	(Woywodt A 2006)

PECAM-1=platelet/endothelial cell adhesion molecule1, ICAM-1=intracellular adhesion molecule 1, VCAM-1=vascular endothelial cellular adhesion molecule 1, ILA/4=inducible by lymphocyte activation/4, MelCAM=melanoma-associated cellular adhesion molecule, Tie-2=angiopoietin-1,2,4 receptor, B7-H3=B& homologue 3, VEGFR-2=vascular endothelial growth factor receptor 2, S=haematopoietic cells, P=platelets, M=monocytes, E=erythrocytes, D=dendritic cells. Expression levels: ++=strong, +=moderate, (+)=weak. [a]Indicates data based on flow cytometric results from authors, [b]Indicates presencew on activated cells.

There is also a huge variation in reported numbers of CECs and CEPs, due to both the diverse nature of the diseases studied and the non-standardised methodology. For example, immunobead and density centrifugation methods tend to show values of around 10 CECs/mL in healthy controls, where as with flow cytometry, numbers are up to 1000 fold increased (Mancusco P 2001; Goon P *et al* 2005). In this study, CEC numbers in healthy controls were 0.45 -1.12 cells per µl of whole blood. Through researching the varying methodologies used previously and through optimisation experiments, it was found that data inconsistencies are possible at any stage in the processing of each sample. The increasingly available automated isolation and staining techniques e.g. CellTracks Autoprep and CellTracks Analyser II system (Immunico Corp, PA, USA), were initially designed to detect circulating tumour cells. These techniques are non-operator dependent, and are fully automated. They can then be followed by gene expression profiling to demonstrate endothelial markers (Smirnov D *et al* 2006; Rowland JL *et al* 2007) These automated techniques are highly costly and only appropriate for larger numbers of samples, and therefore not appropriate for use in this study. However, a recent study published by Ali et al, looked at a group of 76 consecutive breast cancer patients. This study used the automated CellSearch system that allows a more standardised analysis of CECs and showed that baseline CEC levels might be useful in predicting response to chemotherapy (Ali AM *et al* 2011).

The use of flow cytometry requires sequential gating and fluorescence-compensation strategies. This increases inter-laboratory variability and therefore reduces validity of cross-study comaprison. There is extensive variability in CEC values reported in the

literature as a consequence of this (Blann AD *et al* 2005). Another mandatory step in the assay development is validation of the true endothelial origin of cells designated CEC by that assay. For this purpose, several unique features of endothelial cells can be used, including their ability to scavenge acetylated low-density lipoproteins. Other options include the identification of specific surface markers or gene-expression profiling e.g. vascular endothelial cadherin (VE-cadherin), which is present in CECs (Smirnov D *et al* 2006). This was not done as part of the study, since optimisation and proof of concept were the major components of work involved. However, specific validation of CEC and CEP phenotypes is possible and is likely to add weight to findings in larger studies.

This was a small, but unique study. Until this study, CEC and CEP enumeration in stage IV melanoma patients on metronomic versus conventional treatment regimens has not been investigated. It is one of few metronomic schedules that has been used in the treatment of stage IV melanoma and is the only study to be based on dose-escalation schema rather than an empirical percentage of MTD. Whether or not CEC and CEP enumeration can be used for the purpose of prognostic indication will ultimately rest on validation of this methodology in a larger trial (ideally in a substudy to an RCT) with correlation of the biological outcome to clinical endpoints. This testing will always be the vital test of any predictive marker of cancer treatment and CECs-CEPs in melanoma are not exempt. This observational, 'proof of concept' study adds to the expanding body of evidence related to anti-endothelial therapy, not just in melanoma, but in all adult solid malignancies. It is hoped to inspire further work into this field.

Chapter 6

Summary and conclusions

The aims of the *in vitro* study as described in chapter 3 and 4 were:

1. To establish the *in vitro* model, using micro-vascular endothelial cells

2. To test the anti-endothelial and anti-cancer effects of oral chemotherapeutic agents and specific anti-angiogenic agents given in an extended low dosing schedule, given as single-agents

3. To determine the 'metronomic' dose of oral chemotherapeutic agents based on the dose at which significant anti-endothelial effects were seen. Suitable single-agents, at these determined doses, will be taken forward to use in combination with specific anti-angiogenics (described further in chapter 4).

4. Using the *in vitro* MVEC model, to test the potential synergistic effects of combining the multi-kinase inhibitor, sorafenib, with the chemotherapeutic agents selected for this study.

5. Using the *in vitro* MVEC model, to test the potential synergistic effects of the vascular-targetting agent, combretastatin with the chemotherapeutic agents selected for this study.

6. To determine if an additive inhibitory effect on growth was seen in any of the cell lines, when comparing single-agent data to the combination regimens and to rule out combinations that show little or no synergy.

All the chemotherapeutic agents used in this study have good oral bioavailability and are capable of achieving anti-endothelial effects at doses well below MTD. This

makes them potentially suitable for use in future metronomic schedules. Their anti-angiogenic response *in vivo* will be difficult to assess, and will rely on the development of surrogate markers of tumour vasculature.

Throughout this aspect of the study, it became apparent that within the 'empirical metronomic range' there were a number of doses with significant anti-endothelial effect. The MVEC model was optimised in order to ensure that data was reliable and that these effects were reproducible. It was decided, for reasons explained in chapter 3, that the first dose level with highly significant ($p<0.001$) MTT inhibition of MVECs compared to baseline would be taken forward to combination as the 'metronomic dose', provided it did not exceed 15% of MTD (as expected from the published literature range). It was accepted that this in itself was an arbitrary method of establishing a dosing level, but in this respect, there was no guidance in the literature.

In terms of combinations, as described in chapter 4, the etoposide / sorafenib combination showed a significantly enhanced response compared to single agents in both the MVEC and SK-MEL-2 cell lines by over 55% (see **figure 4.4**). Of all the sorafenib-chemotherapy combinations, this combination showed the greatest anti-endothelial synergistic effect. This was greater than the effect seen in both the temozolomide and paclitaxel combinations. This suggests that the combination may potentially warrant future study in both the *in vivo* and clinical setting. The effects of combining combretastatin, a non-specific anti-angiogenic agent, were not as successful as that observed with a specifically targeted agent such as sorafenib. This may be supporting evidence for its lack of efficacy in such combinations when used

in the *in vivo* setting.

In spite of the limitations of an *in vitro* model such as this, the study yielded results which compared favourably with other published works on *in vitro* dosing of metronomic chemotherapy (Lam T *et al* 2007). With an *in vitro* model, it is impossible to assess pathophysiological mechanisms of action, and therefore further *in vivo* work would be required to assess efficacy of combinations. This study does not further our understanding of the mechanisms of action of these agents, nor does it replace or even reflect the clinical setting. It is however hoped that preliminary *in vitro* studies such as these, can provide a logical starting point for initiating *in vivo* and phase I studies. They may also aid in preventing phase I trials, based on empiricism, from being undertaken. Since combinatorial options are huge, alternative, potentially more effectual options are plentiful, provided they are at least given the chance at an *in vitro* level first. The aims of this *in vitro* study, as laid out above, were therefore achieved.

It is hoped that future work from this study can use this screening tool to produce patterns of synergistic responses between agents and eventually move forward to determine changes in down-stream proteins that are pertinent to specific agents. Analysis of the proteome may well prove to uncover unknown targets of both specific and non-specific agents. This may also provide further justification for tailor-made regimens designed for specific patients and tumours, aiming for maximum efficacy rather than maximum cytotoxicity.

The aims of the clinical study as described in chapter 5 were:

1.To test methodology and to optimise the technique of isolating CECs and CEPs from whole blood.

2.To isolate CECs and CEPs from whole blood samples of patients recruited into the scientific sub-study (as described in chapter 2).

3. To compare CEC and CEP levels between patients on metronomically-dosed and conventionally-dosed chemotherapy.

This study was a very small, but unique study. Until this study, CEC and CEP enumeration in stage IV melanoma patients on metronomic versus conventional treatment regimens has not been investigated. It is one of few metronomic schedules that has been used in the treatment of stage IV melanoma and is the only study to be based on dose-escalation schema rather than an empirical percentage of MTD. Considering the mechanism of action of metronomic regimens and of antiangiogenic inhibitors, one of the most important problems faced is the choice of the OBD. Tumour stabilisation rather than objective response is probably a more appropriate endpoint, as well as TTP (Gasparini G *et al* 2005; Jubb A *et al* 2006).

Whether or not CEC and CEP enumeration can be used for the purpose of defining the OBD or as prognostic indicators will ultimately rest on validation of this methodology in a larger trial (ideally in a substudy to an RCT) with correlation of the biological outcome to clinical endpoints. There are a number of unresolved questions on the determination of CECs and CEPs: a) the sensitivity and reproducibility of the methods used; b) the challenge if tumours may mobilise sufficient CEPs to even be detected in clinical practice; c) the best antigen panel for the characterisation of these cells.

The aims of this observational, 'proof of concept' study have been achieved. The results could have been enhanced in a number of ways, as discussed in chapter 5, and in particular, by employing additional verification techniques, including use of further cellular markers of identification. It is work that adds to the expanding body of evidence related to anti-endothelial therapy, not just in melanoma, but in all adult solid malignancies. It is hoped that further work into this field will focus on further stream-lining of methodology along with accurate verification. Assessment of these potential biomarkers in trials that employ the combinatorial, metronomic approach has already showed promise. Malignant melanoma trials adopting this approach, along with use of specific anti-angiogenic agents should aim to assess CECs and CEPs in a larger cohort of patients, ideally comparing groups with and without a specific anti-angiogenic agent.

In conclusion, this thesis has focussed on novel anti-endothelial strategies in malignant melanoma and the principles and applications of metronomic chemotherapy. The *in vitro* aspect of this study has promise to be used as a pre-*in vivo* screening tool in assessing potential compound synergy. It may also assist in excluding futile combinations. The CEC / CEP isolation study has generated a hypothesis for the use of these cells as biomarkers when adopting anti-angiogenic and metronomic strategies in malignant melanoma. Ideas for future work in both these aspects of the study have been suggested.

Considering the complex angiogenesis regulatory network that involves multiple angiogenic factors produced by various cell types, any antiangiogenic therapy aimed

at a single angiogenic factor is unlikely to be effective, as laid out in this thesis. Metronomic chemotherapy can provide an ideal way of administering chemotherapy in combination with many different types of biologic targeted therapies, antiangiogenic agents, signal-transduction inhibitors (e.g trastuzumab), aromatase inhibitors (e.g. letrozole), COX-2 inhibitors (e.g. celecoxib) and immunotherapeutic tumour vaccines.

Throughout the completion of this thesis, my own views on the future of cancer therapy have been changed. It seems to me that unless molecularly-targeted approaches continue to be enhanced and pursued, drug-based cancer treatment as we know it will continue in a state of mediocracy. Logical dosing strategies rather than empiricism have been shown to have a greater patient benefit in terms of quality of life and lower toxicity profiles. The conventional mass cytotoxic approach is both out-dated and unscientific. Molecularly-targeted, tumour-specfic cocktails designed on an individual patient basis may well be the future of modern oncological treatment.

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Appendices

Appendix 1: Media and Reagents used in MVEC experiment

MVECs - Medium 199 (500mL) with Earle's salts**,** L-glutamine (5mL) and sodium

bicarbonate (Sigma, U.K.)

25mL microvascular endothelial growth supplement (TCS Cellworks Ltd, U.K.)

5mL antibiotic (gentamicin/amphotericinB) supplement (TCS Cellworks Ltd, U.K.).

Cancer cells - RPMI 1640 Medium (500mL) with L-Glutamine (5mL)

100 U/ml Penicillin and 100μg/ml Streptomycin (Sigma, UK) (5mL)

10% (v/v) HiFBS (50mL)

Freeze medium – MVECs

20% (v/v) BSA – 10mL

10% (v/v) Dimethyl-Sulfoxide (DMSO) (Sigma, UK) – 5mL

70% MVEC Medium 199 - 30mL + 'antibiotics + growth factors' TCS Cellworks Ltd

– 5mL

Freeze medium – cancer cells

40% (v/v) HiFBS – 20mL

10% (v/v) Dimethyl-Sulfoxide (DMSO) (Sigma, UK) – 5mL

50% RPMI 1640 Medium with L-Glutamine + antibiotics – 25mL

Phosphate Buffered Saline (PBS) pH 7.2 (1L)

5.84g NaCl

4.72g Na_2HPO_4

2.64g $NaH_2PO_4.2H_2O$

made up to 1L with distilled water

Lysis Buffer

Dimethylformamide-water,44:56 v/v containing 0.3M sodium dodecyl sulfate, pH 6.7

MTT

250mg thiazolyl blue tetrazolium bromide

50mL sterile distilled water

filtered, wrapped in foil and stored in refrigerator (light-sensitive)

Attachment factor for MVECs

0.1% (w/v) gelatin in sterile phosphate buffered saline

TrypLE™ Select (Invitrogen)

1x concentration of recombinant enzyme 'rProtease' in PBS with 1mM EDTA

PBS/0.25% (w/v) BSA/Azide (500ml) for FACS

1.25g BSA

3.12ml of 10% (w/v) sodium azide (NaN_3) made up to 500ml with sterile PBS

Freeze medium for PBMCs

90% fetal bovine serum – 45mL and 10% dimethyl sulfoxide (DMSO)-5mL

Thaw medium for PBMCs

90% RPMI + 10% FBS

MACS Buffer (kept at 2-8^{0}C)

500mL phosphate-buffered saline (PBS)

0.5% bovine serum albumin (BSA) – 250mg

2mM EDTA – 0.6g

FACS Buffer

500mL of phosphate buffered saline (PBS)

1.25g bovine serum albumin (BSA) and 3.12mL Azide

FcR Blocking Reagent (kept at 2-8°C) contains PBS, pH 7.2, 0.5% BSA and 2mM

EDTA

Appendix 2.1: Information sheet for study patients

**A new way of monitoring and predicting the severity of melanoma
by measuring specific cells.**

PATIENT INFORMATION LEAFLET

I would like to invite you to take part in a research study. It would be much appreciated if you could read the following information to understand why the research is being done and what it involves. I will be happy to explain anything that you do not understand. Please take as much time as you need to read the information and make a decision.

What is the purpose of this study?

We have been trying to develop better tests that might help to monitor and predict the outcome in patients with melanoma. This study aims to develop a test to isolate three different types of cells from the blood stream. The actual numbers of these cells may reflect the severity of melanoma.

We already know that there are melanoma cells circulating in the blood stream of some patients with the disease. There are also two other types of cells which have been previously shown to increase in the blood stream in certain tumours and diseases. These two cell types (endothelial) are the building-blocks of new blood vessels. New blood vessels are made in most tumours in order that growth and spread can be sustained. Therefore quantifying these cells may well be a useful tool when looking at growth and spread of melanoma.

Measuring these three cell types may also help to monitor the effectiveness of chemotherapy i.e. when treatment is working, cell numbers go down and vice versa.

To carry out this study and measure these cells, it is necessary to collect three blood samples. We are therefore inviting you and other patients who are also due to start chemotherapy to take part in this melanoma study. This would require three samples of blood to be taken; one before chemotherapy, one during and one after chemotherapy.

Why have you been chosen?
As you are aware, you have been diagnosed as having a type of skin cancer called malignant melanoma. Studies have shown that there is a significant risk that melanoma can seriously shorten life if it has spread elsewhere in the body or if it has reoccurred. Your oncology doctor has recommended that you start treatment with a combination of chemotherapy drugs to try to reduce the chance of the disease spreading any further.

What will happen if you decide to take part?

If you decide to take part in this study, **your treatment plan would not be affected**. We would like to collect three 50 ml blood samples (each a small cup full) during your usual outpatient clinic visits if you agree to participate. This would be done at the same time as the routine blood tests are taken, so there would be **no extra needle-sticks or hospital visits required.**

If you choose to participate, your involvement in the study will be complete at around eight weeks after your chemotherapy has finished. **Further blood tests for research purposes after this will not be necessary.** You may however need to continue having monitoring blood tests for the melanoma and **not** for research.

If you are willing, we would like to continue to review your notes regularly over the next few years to record any data that is relevant to your diagnosis and treatment.

What do you have to do?

Other than having these three blood tests taken, you do not have to do anything different to patients not involved in the study.

Do you have to take part?

No, of course not. Your participation is a matter of your choice.
Your treatment will not be affected whether you <u>do</u> or <u>do not</u> take part.

Are there any risks involved?

No. There are no risks to you.

Are there any benefits of taking part?

There will not be any clinical benefit to you, but we hope that it will benefit patients with melanoma in the future.

What if something goes wrong?

If, for any reason, you are unhappy with the treatment you receive by any member of staff, you should, of course, follow the routine hospital complaints procedure. Your legal rights for claiming compensation for **any** injury where you can prove negligence is not affected.

Confidentiality

Details of yourself, your present and past treatment as well as your participation in the study will be kept in the strictest confidence. These details will be recorded in a case file to be retained by the Plastic Surgery Department and on a protected database for the duration of the research

study - anticipated to last approximately two years. A copy of your consent form for this study will also be kept with this information.

What will happen to the results of the research study?

The results of this research will be completely confidential, and no personal details will be divulged. These results will be analysed and written in my research degree thesis (MD). The findings may be presented at learned societies or published in scientific journals. In such cases the information will only identify you with a number and **not** your name or other personal details.

We would like to thank you for your attention so far and hopefully for your participation in this study.

By signing the accompanying **consent** form you will be giving your agreement to be entered into this research study.

Your rights
Your participation in this study is voluntary. If you decide not to enter the study your treatment will not be affected.

Who is organising and funding the research?
This research study is organised jointly by
Dr Alexandra Murray

In association with:
THE PLASTIC SURGERY DEPARTMENT
(Castle Hill Hospital, Cottingham)
Mr PRW Stanley, Mr N Hart, Mr A Platt, Mr M Riaz

THE ACADEMIC ONCOLOGY DEPARTMENT
(Princess Royal Hospital, Hull)
Dr A Maraveyas
The doctors conducting this study are **not** being paid to include you in this study.

Contact for further information.
Please feel free to contact the research doctor - Miss Alexandra Murray, if you have any queries.

The Department of Plastic Surgery,

Castle Hill Hospital, Castle Road, Cottingham, Hull, HU16 5JQ.

Tel: (01482) 875875 ext 2707

Mobile: 07779727984

Appendix 2.2: Consent form for study patients

<u>CONSENT FORM - Version 1 (2/8/2006)</u>

A new way of monitoring and predicting the severity of melanoma by measuring specific cells.

Principle Investigator/Researcher: **Dr Alexandra Murray**

<Patients Name and Address>

………………………………..

………………………………..

……………………………….. **Please initial boxes**

I confirm that I have read and understood the patient information sheet dated (2/8/2006 version 1) for the above study and have had the opportunity to ask questions.

☐

I understand that details of my current melanoma treatment will be examined as part of the above study and may be viewed by responsible members of the research team. I give my permission for these individuals to have access to my records.

☐

I also give consent for storage of clinical information about me on a password protected computer-database for the duration of the study. I understand that this information will be coded and anonymous.

☐

I give permission for 3 samples of blood to be taken   ☐

I understand that my participation is voluntary   ☐

I agree to take part in the above study   ☐

_____   _____   _____
       Name of Patient   Signature   Date

_____   _____   _____
   Name of Research Doctor   Signature   Date

_____   _____   _____
  Name of interpreter (if required)   Signature   Date

Appendix 2.3: Information sheet for healthy volunteers

A new way of monitoring and predicting severity of melanoma by measuring specific cells.

VOLUNTEER INFORMATION LEAFLET

I would like to invite you to take part in a research study. It would be much appreciated if you could read the following information to understand why the research is being done and what it involves. I will be happy to explain anything that you do not understand. Please take as much time as you need to read the information and make a decision. *There is absolutely no obligation to participate. If you decide not to participate now then please feel free to do so. If you decide not to participate after reading this information sheet, then that is not a problem at all. Your time and effort is much appreciated.*

What is the purpose of this study?

We have been trying to develop better tests that might help to monitor and predict the outcome in patients with melanoma. This study aims to develop a test to isolate three different types of cells from the blood stream. The actual numbers of these cells may reflect the severity of melanoma.

We already know that there are melanoma cells circulating in the blood stream of some patients with the disease. There are also two other types of cells which have been previously shown to increase in the blood stream in certain tumours and diseases. These two cell types (endothelial) are the building-blocks of new blood vessels. New blood vessels are made in most tumours in order that growth and spread can be sustained. Therefore quantifying these cells may well be a useful tool when looking at growth and spread of melanoma.

Measuring these three cell types may also help to monitor the effectiveness of chemotherapy i.e. when treatment is working, cell numbers go down and vice versa.

We are therefore inviting you and other volunteers to give one sample of blood to test for these cell types. It is essential that we have a control group of healthy volunteer blood samples against which to compare patient blood samples. This will highlight any technical errors with cell pick-up rate and strengthen our research.

What will happen if you decide to take part?

We would like to collect one 50 ml blood sample (a small cup full) at a time convenient to you, if you agree to participate. *There is absolutely no obligation to participate, and this is purely a voluntary study.*

If you choose to participate, your involvement in the study will be complete after this one sample has been taken.

Do you have to take part?
No. _You do not have to take part at all_.
Are there any risks involved?
There is always the possibility of a little bleeding or bruising after having a blood test.
Are there any benefits of taking part?
There will not be any immediate clinical benefit to patients with melanoma, but we hope that it will benefit patients in the future.
Confidentiality
Details of yourself and your participation in the study will be kept in the strictest confidence. These details will be recorded in a case file to be retained by the Plastic Surgery Department and on a protected database for the duration of the research study - anticipated to last approximately two years. A copy of your consent form for this study will also be kept with this information.

What will happen to the results of the research study?
The results of this research will be analysed and written up (anonymously) in my research degree thesis (MD). The findings may be presented at learned societies or published in scientific journals. In such cases the information will only identify you with a number and not your name or other personal details.
We will keep you informed of any positive findings from the study.

By signing the accompanying **consent** form you will be giving your agreement to be entered into this research study.

Your rights

Your participation in this study is completely voluntary _– there is no obligation to take part_.

Who is organising and funding the research?

This research study is organised jointly by
Miss Alexandra Murray (07779727984)
In association with:
THE PLASTIC SURGERY DEPARTMENT
(Castle Hill Hospital, Cottingham)
Mr PRW Stanley, Mr N Hart, Mr A Platt
THE ACADEMIC ONCOLOGY DEPARTMENT
(Princess Royal Hospital, Hull)
Dr A Maraveyas

The doctors conducting this study are **not** being paid to include you in this study.

Appendix 2.4: Consent form for healthy volunteers

<u>CONSENT FORM – Version 1 (2/8/2006)</u>

A new way of monitoring and predicting the severity of melanoma by measuring specific cells.

Principle Investigator/Researcher: **Miss Alexandra Murray**

<Patients Name and Address>

…………………………………..

…………………………………..

…………………………………..   **Please initial boxes**

I confirm that I have read and understood the volunteer information sheet dated (Version 1 2/8/2006) for the above study and have had the opportunity to ask questions.

☐

I give consent for the storage of information gained from my blood sample to be stored on a password protected computer-database for the duration of the study. I understand that this information will be coded and anonymous.

☐

I give permission for one sample (50ml) of blood to be taken   ☐

I understand that my participation is entirely voluntary   ☐

I agree to take part in the above study   ☐

_____   _____   _____
Name of volunteer   Signature   Date

_____   _____   _____
 Name of research doctor   Signature   Date

_____   _____   _____
 Name of interpreter (if required)   Signature   Date

Appendix 3: Statistical analysis for clinical study (chapter 5)

Descriptive statistics for the numbers of CEC/µl of whole blood

Patient group	Sample size	Sample	Kolmogorov-Smirnov test	Mean	Standard deviation
Control (Healthy)	4	Baseline	Z = .604 p = .859 [ns]	0.86	0.293
Conventional	4	Pre-treatment	Z = .532 p = .940 [ns]	14.01	4.349
		Mid-treatment	Z = .518 p = .952 [ns]	9.55	3.073
		Post-treatment	Z = .462 p = .983 [ns]	11.86	6.945
Metronomic	4	Pre-treatment	Z = .475 p = .988 [ns]	6.55	3.596
		Mid-treatment	Z = .518 p = .952 [ns]	11.25	2.449
		Post-treatment	Z = .500 p = .964 [ns]	11.49	4.987

Descriptive statistics for the numbers of CEP/ µl of whole blood

Pt group	Sample size	Sample	Kolmogorov-Smirnov test	Mean	Standard deviation
Control (Healthy)	4	Baseline	Z = .729 p = .662 [ns]	0.005	0.010
Conventional	4	Pre-treatment	Z = .651 p = .791 [ns]	0.35	0.224
		Mid-treatment	Z = .647 p = .796 [ns]	0.29	0.020
		Post-treatment	Z = .670 p = .761 [ns]	0.56	0.221
Metronomic	4	Pre-treatment	Z = .773 p = .589 [ns]	0.22	0.273
		Mid-treatment	Z = .511	0.62	0.726

		$p = .956$ [ns]		
Post-treatment		$Z = .541$	0.42	0.409
		$p = .932$ [ns]		

[ns] No significant deviation from normality at $p = .05$

Kolmogorov-Smirnov tests indicated that the distributions of the estimates of CEC and CEP did not deviate from normality justifying the use of parametric statistics.

Results of One-way ANOVA to compare the baseline (pre-treatment) mean CEC/µl in the three groups of patients

	Sum of Squares	Degrees Freedom	Mean Square	Variance ratio F	Significance p	Effect Size Eta ^{2}
Between groups	347.635	2	173.818	16.328	.00	.784
Within groups	95.807	9	10.645			
Total	443.442	11				

Welch's Statistic	Degrees of Freedom		Significance p
19.799	2	4.04	.008*

* Significant difference at $p = .05$

Results of Dunnet's T3 post-hoc test for multiple comparison of the mean CEC/µl estimates in the control and treatment groups

Comparison of means		Mean Difference	Significance p
Control (Healthy)	Treatment regimen 1	-13.14	.021*
	Treatment regimen 2	-5.69	.115[ns]
Treatment regime 1	Treatment regimen2	7.45	.103[ns]

* Significant difference at $p = .05$.   [ns] No significant difference at $p = .05$

Appendix 4: Case report

This is the case report of 'patient 4' on conventional treatment discussed in chapter 5.

A 70 year old female was referred to the skin cancer multi-disciplinary team following excision of a lesion from the right lower flank area in March 2007. This lesion was shown by the referring hospital to be highly malignant with greater than 60% proliferative index and brisk mitotic activity including atypical mitoses. S100 positivity suggested the strong possibility of melanoma. Complete excision was not possible to be commented on, nor was there a possibility of commenting on whether this was a primary or secondary lesion. A wide local excision and sentinel node biopsy was carried out by the local plastic surgery department early in May 2007. There were also reports that the patient had experienced some symptoms of pain in the right pelvic area since March 2007. Plain X-ray was carried out to reveal a lytic lesion in the right inferior pubic ramus, associated with a pathological fracture.

CT scan taken in May 2007 preoperatively revealed a large right flank mass measuring 4.2x2.6cm in the actual plane. A Trucut biopsy of this lesion was shown histologically to be metastatic melanoma. The patient was then referred for radiotherapy to the right symphysis pubis, 20 Gy in 5 fractions.

In view of progressive disease, the patient consented to start on dacarbazine and vindesine chemotherapy. At this point, the patient also consented to participate in the scientific sub-study for enumeration of CECs and CEPs. She remained relatively well throughout treatment on the conventional regimen.

CT scan revealed stable apprearances of the pubic ramus in April 2008. In July 2008,

there were signs of bony healing, and some atypical nodular changes in the lower lungs, but no clear evidence of metastases. CT scan in July 2009 revealed no change and stable disease. She therefore remained stable for 2 years. However, unfortunately in May 2010, further metastatic deposits were noted and disease progressed rapidly.

*Additional signed informed consent was gained from the patient for use of this case report in my thesis.