TITLE: Driving tumours to become their own vaccine site: assessing the therapeutic and immunological consequences of anti-vascular agents in cancer

ABSTRACT

Mesothelioma and lung cancer are diseases that cannot be cured. The need to better understand these neoplastic diseases is imperative since current standard treatments alleviate pain or only increase the survival period by a few months. The tumour microenvironment plays a major role in tumour growth and treatment efficacy. Two key local regulatory features are tumour infiltrating immune cells and the process of angiogenesis. Modulating the tumour microenvironment may contribute to improving therapy outcomes. Innate and adaptive immune responses recognise tumour cells; some of which may slow tumour growth (effector cells) whilst others contribute to tumour development through the production of angiogenic and immunosuppressive factor (suppressor cells). Changing the balance of effector/suppressor cells in the tumour microenvironment by rendering tumour blood vessels to be more permissive to effector cell traffic may be a successful strategy to eradicate tumours. This study will evaluate the therapeutic and immunological consequences of anti-vascular agents in cancer aiming to provide a better understanding of anti-cancer immune responses.

OBJECTIVES

Hypothesis: Therapies that modify tumour-associated blood vessels to become permissive to immune cell traffic will be more successful than those that do not.

Aim #1: To assess the effect of vascular-targeting agents on lung cancer and malignant mesothelioma tumour growth using mouse models.

Aim #2: To determine the immunological impact of anti-vascular agents when used to treat cancer.

Aim #3: To determine the anti-cancer potential of combining vascular-targeting agents with immunotherapy.

BACKGROUND

Malignant mesothelioma and lung cancer

Malignant mesothelioma (MM), caused by asbestos exposure, is an aggressive tumour that arises from the abnormal proliferation of mesothelial cells of the serosa membrane (pericardium, peritoneum, and pleura). Whilst the disease is relatively rare the numbers of cases are expected to rise on account of the long latency period post asbestos exposure that results in tumours resistant to standard therapy (1-3). Once clinical symptoms manifest, the median survival period of a MM patient is less than 12 months; fewer than 5 per cent of individuals survive five years (2). Current treatment options such as chemotherapy, radiotherapy, and surgery only prolong life expectancy by a few months therefore new treatment approaches are desperately needed (1, 2, 4). Following inhalation or ingestion asbestos fibres penetrate serous membranes to cause inflammation and irritation. Malignancy results when fibres in the mesothelial cell layer contribute to the production of toxic components such as reactive oxygen metabolites (5) generating molecular changes in one or more mesothelial cell (e.g. DNA point mutations and chromosomal breaks) (6). The most common molecular change is the presence of a single copy of chromosome 22. Mutations, include deletions in the 1p, 3p, 9p, and 6q chromosome arms. In addition, changes have been seen in the tumour-suppressor genes p16 (CDKN2A) and p14 (ARF) (6).

The term ‘lung cancer’ includes all malignant tumours that arise from the bronchial, bronchiolar, or alveolar epithelium (7). Lung cancer is the most common cause of mortality

from cancer and 17.8% of lung cancer patients survive for five years after diagnosis of the disease (8). Lung cancer displays a significant range of molecular heterogeneity within individual patients (9). Histologically, lung cancer is classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). In approximately 90% of lung cancer cases, smoking is the major factor that accounts for the development of the disease (10). Other causes, including environmental exposure such as radon, asbestos, air pollution and chromium, have been found to be associated with increased risk for lung cancer (7).

Standard treatment

MM ***Chemotherapy***: Chemotherapy (cisplatin and pemetrexed), the standard care offered to MM patients, only extends life expectancy by a few months and is not curative (1,4). Quality of life may be an issue as symptoms such as breathlessness and pain can increase during treatment (1). Importantly chemotherapy may not be as detrimental to the immune system as first thought and its combination with immunotherapy has shown promise in preclinical animal studies and in clinical trials model (discussed below).

Surgery: Surgical intervention is mainly used for palliation (11). It has been proposed that debulking surgery or radical resection should be followed by adjuvant treatment to eliminate residual disease (1). In some cases, surgery raised the median survival to more than 2 years (12).

Radiotherapy: Radiotherapy is often only used for palliation and is not otherwise useful (13).

Lung Cancer: Surgical resection is the proposed treatment for patients with stage I or II NSCLC. The major cause of death in lung cancer patients who undergo to complete surgical resection is a distant recurrence that usually takes place within five years following treatment (7). Comparatively, aggressive chemotherapy combined with radiotherapy shows an increase in survival rates in 20% to 25% of patients with stage III, locally advanced NSCLC, or limited-stage SCLC. Chemotherapy can help patients survive for two further years (14) and palliate symptoms of incurable diseases (stage IV or metastatic NSCLC and extensive-stage SCLC) (7, 10).

Angiogenesis and its role in tumour progression and immunity

Angiogenesis, also known as neovascularisation, is a complicated process by which new blood vessels develop via the proliferation of endothelial cells and extensions from the existing vasculature (15). Angiogenesis is a biological process essential to the developing foetus, normal growth, and tissue repair (16). In the case of a tumour, the angiogenic process is described as the formation of a network of blood vessels that enters the tumour mass to provide cancerous cells with the nutrients and oxygen they need, and to remove waste products (17). This environment, when available, leads to tumour growth, and metastasis (18). Tumour cells exploit their microenvironment to grow beyond a size of 1–2 mm^3; in this way, hypoxia and nutrient deprivation result from excessive tumour lesions, and induce the angiogenic pathway (19, 20). Disorganised angiogenesis is considered a hallmark of cancer development (16). Jain and colleagues (2008) describe the appearance and functional abnormalities of blood vessels within a tumour. Many abnormalities arise from an imbalance of pro- and anti-angiogenic signalling within tumours, which contribute to the formation of an unusual blood vessel network characterised by dilated, tortuous, and hyper-permeable vessels (16, 17). Dysfunctional vessels associated with physiological consequences include those with temporal and spatial heterogeneity in tumour blood flow, hypoxia, high acidity, and increased tumour interstitial fluid pressure (17).

Several angiogenic factors that support and induce angiogenesis in MM and lung cancer have been found to be secreted from tumour-associated inflammatory cells, the extracellular matrix, or tumour cells, including Vascular endothelial growth factor (VEGF), basic fibroblast growth factor, Transforming growth factor beta (TGF-β), hepatocyte growth factor/scatter factor (HGF/SF), platelet-derived growth factors (PDGFs), insulin-like growth factor-I (IGF-I), and many cytokines such as Interleukin(IL)-6 and IL-8 (21-23). Their actions on tumour angiogenesis manifest in the positive regulation of endothelial cell proliferation, migration, and vascular permeability (24).

Negative consequences from vessel abnormalities include a chaotic layout and a high number of aberrations, such as increased blood vessel diameter leading to irregular blood flow. Blood flow might be static at one site but brisk at another; moreover, blood flow may suddenly reverse. Such random blood-flow patterns make uniform drug delivery a challenge. Unusually large pore sizes between the endothelial cells that form the vessel wall and a high interstitial fluid pressure can block oxygen from transmitting to tumour tissue and molecules designed to penetrate the vasculature. Furthermore, dysfunctional blood vessels create changes in the tumour microenvironment by producing a hypoxic environment that features high levels of acidity. The efficiency of some therapies such as radiation, and specific types of chemotherapy that require oxygen to kill cancerous cells is reduced. Additionally, a hypoxic environment changes gene activity in such a way as to help cancerous cells migrate toward healthy tissue. The ability of immune cells to kill cancerous cells is countered by the acidic environment. Finally, growing tumours feature fluid build-up that leads to swelling and painful symptoms; the risk of metastasis is higher wherever fluid pressure pushes tumour-generated proteins and cancerous cells toward healthy tissues and into the lymphatic vessels (25). In addition to the role of tumour angiogenesis in supporting the tumour, the angiogenic switch affects leukocyte extravasation into the tumour thereby presenting a major obstacle for successful tumour immune therapy (26, 27).

Angiogenesis signalling cascade and role of vegf

Several angiogenic factors have been reported to promote the angiogenesis signalling cascade of MM and lung cancer, such as IGF-I, PDGFs, and VEGF. Among these, VEGF is the most powerful angiogenic factor, and plays a key role in MM biology (28). A study conducted on 37 patients with pleural MM showed that 36 of 37 MM samples produced positive VEGF stains (29). VEGF is a signal protein that stimulates cellular responses by binding to the tyrosine kinase receptors VEGFR-1 (flt-1) and VEGFR-2 (Flk-1/KDR) on the surface of endothelial cells (8, 29), and malignant cells within MM tumours express VEGF and both VEGFR-1 and 2. High-affinity binding of VEGF with VEGFR-2 triggers endothelial cell proliferation (30). Furthermore, cellular responses including proliferation are activated when VEGF induces VEGFR-2 autophosphorylation in MM cells (31). Monocytic cells express only VEGFR-1 on the surface, and VEGF induces their migration and production of tissue factor such as matrix metalloproteinase 9 (MMP-9). VEGFR-1 receptors also mediate the production of MMP-1, -3, and -9 in smooth muscle cells (31). Therefore, the binding of VEGF to VEGFR-1 and 2 expressed by tumour cells is followed by several processes that contribute to tumour progression, including the stimulation of tumour cell proliferation, the breakdown of extracellular matrix, and tumour cell migration. Pathological angiogenesis in tumours results from an imbalance between anti- and pro-angiogenic factors, therefore restoring the balance may revert blood vessels back to the normal state and slow tumour development.

Tumours and the immune system

The immune system defends the body against infection by pathogenic viruses, bacteria and fungi, and against abnormal cells that could develop into cancer. It comprises of innate and adaptive immunity. Innate immunity consists of different cells, including macrophages, dendritic cells, and polymorphonuclear neutrophils. Adaptive immunity consists of cell mediated immunity (T-cells) and humoral immunity (B-cells).

Innate Immunity

Neutrophils: Neutrophils are potent, polymorphonuclear leukocytes with strong antimicrobial and inflammatory capacities. Their recruitment to a site of inflammation, including a tumour site, is mediated by specific chemoattractant mediators secreted by tumour cells or stromal cells, including infiltrating immune cells. IL-8/CXCL8 is a highly effective neutrophil chemoattractant via binding to CXCR1 and CXCR2 receptors (32). A large number of tumour-associated neutrophils (TANs) can infiltrate tumours (33, 34). Studies on multiple tumour types demonstrated that specific neutrophil subsets are associated with tumours. TAN subsets can be classified as either pro-inflammatory N1 that inhibit tumour growth or anti-inflammatory tumour promoting N2 cells (35). Different studies have proven that tumour-mediated signals like TGF-β induce N2 subsets (34). N2 cells promote immunosuppression, tumour angiogenesis, and metastasis, and represent the majority of TANs. N2 cells promote immunosuppression via production of arginase, which deactivates T-effector cell functions including proliferation and responsiveness, (36) and production of MMP-9 and VEGF (37). MMP-9, secreted by neutrophils, degrades the extracellular matrix and prevents tumour cell apoptosis, inducing VEGF secretion (34,38). N2 cells support tumour metastasis via collagenase-IV and heparanase production that degrade the basement membrane, thus promoting tumour cell extravasation (34). A study of lung cancer and mesothelioma conducted using a murine model showed that depletion of N2 neutrophils results in tumour growth inhibition, even in the absence of $CD8^+$ T cells (35, 36, 38). In contrast, N1 cells are pro-inflammatory and anti-tumourigenic and have been shown to promote tumour cell death through direct cytotoxicity, contributing to tumour regression (36). However, TGF-β in the tumour microenvironment diminishes the generation of N1 cells (36). The absence of TGF-β causes N1 cells to produce several cytotoxic mediators, including tumour necrosis factor alpha (TNF-α), macrophage inflammatory protein 1α , hydrogen peroxide , and nitric oxide (NO) (39). N1 cells have been shown to cause oxidative damage to tumour cells by secreting reactive oxygen species in mice-bearing mesothelioma tumours (36). Furthermore, secretion of high levels of collagenase-2 by N1 cells is protective in Lewis lung carcinoma (40).

Macrophages: The tumour microenvironment of most solid tumours including mesothelioma and lung cancer contains an abundance of macrophages which represent the majority of tumour-associated immune cells and can reach up to 50 % of a malignant tumour mass (41). The origin of tumour-associated macrophages (TAMs) is not clear. One theory is that immature bone marrow-derived monocytes migrate through the blood into the tumour site to develop into mature macrophages (42). The other theory is that local tissue macrophages are recruited into the tumour site (43). Similar to TANs, TAMs have been divided into two classifications, pro-inflammatory M1 and anti-inflammatory M2. There is evidence that M1 cells are formed at the early stages of tumour development. M1 cells play direct and indirect anti-tumoural roles. They do so directly by releasing cytotoxic substances, such as reactive oxygen species (ROS), nitrogen intermediates, granulocyte/macrophage colony-stimulating factor, TNF-α, macrophage migration-inhibitory factor (MIF), and IL-12. They act indirectly through the activation of other immune cells, such as Natural killer cells and T-cells, to

secrete soluble molecules such as interferon-γ (IFNγ) and IL-6. However, M1 cells may damage tissue directly and cause DNA mutation, as well as supporting cell survival and tumourigenesis via the production of particular mutagens, such as ROS and cytokines (e.g., MIF, TNF-α, and IL-6) (7). On the other hand, M2 macrophages are reported to dominate in advanced tumour stages, possibly by 'reprogramming' in response to local factors such as IL-4 and IL-13. M2 cells support tumour growth, metastasis, angiogenesis, and suppress the immune response through secretion of IL-10, NO, prostaglandin E2, reactive oxygen, and high levels of TGF-β (44). M2 TAMs are found in abundance in tumour hypoxic regions, where they release factors, such as VEGF, and angiogenesis-modulation enzymes, such as MMP-9 (45).

Dendritic Cells: Dendritic cells (DCs) are potent antigen-presenting innate immune cells that initiate adaptive immune responses against pathogenic infections, as well as eliciting anti-tumour immunity (46). DCs can be classified according to their early differentiation into myeloid and lymphoid progenitors. In humans, further subsets were recognized in the blood where myeloid DCs include $CD11c^{+}/CD123^{dim}$ and lymphoid DCs include $CD11c^{-}/CD123^{+}$ with overlapping but different properties (47). In the mouse, DCs can be classified into conventional DC and plasmacytoid DC; both express CD11c and major histocompatibility complex-II (MHC-II), but differ in their expression of CD4 and CD8α (Table.1) (48). DCs are the key initiator of anti-cancer adaptive immune responses and play a role in the induction of tumour antigen-specific MHC-restricted T-cells. DCs recognize abnormal cells, such as cancer cells, and take up and process cancer-related antigens at the tumour site, before they migrate to draining lymph nodes. During this process they mature and may become potent activators of T-naïve cells (49). DC maturation and activation is determined by interactions between danger-associated molecular patterns on damaged tissues and tumour cells and pattern-recognition receptors on DCs (50). An example of the DC activation process is demonstrated by the interaction between high-mobility group box 1 released from tumour cells and Toll-like receptor-4 expressed by DCs (51). DCs may encounter several factors that prevent them from inducing functional T cells. The most common tumour cell-derived factors that affect DC function include IL-10, IL-6 and macrophage colony stimulating factor (MCSF) with the latter inhibiting the differentiation of DCs from $CD34^{+}$ progenitors. VEGF release from tumours can alter DC maturation, and high serum levels of VEGF have been associated with the presence of immature myeloid cells in the blood, which closely correlated with the stage and duration of clinical neoplastic disease (52).

Adaptive Immunity

T-Lymphocytes: T-cells develop from hematopoietic stem cells located in the bone marrow. However, they mature in the thymus generating $CD4^{+}$ T-cells and $CD8^{+}$ T-cells (53). T-cells identify tumour cells through binding to peptides presented in MHC molecules. $CD4^{+}$ T-helper cells consist of three main subsets: T_H1, T_H2, and T_H17. T_H1 cells activate $CD8^{+}$ T-cells. T_H2 cells promote humoral immunity via production cytokines such as IL-4 that provide help to B cells (54). The T_H17 subset enhances high-level production of TGF-β and IL-6 (55) and plays a role in infection and tumour immunity. Another subset of $CD4^{+}$ T cells is known as regulatory T cells (Tregs). Tregs can be differentiated from activated $CD4^{+}$ T cells by the expression of Forkhead box 3 (Foxp3) within tumours (9). Approximately 5% to 10% of the $CD4^{+}$ T cell population comprises $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells that are naturally present in the immune system (56, 57). Typically, an abundance of tumour-specific Treg cells is considered a poor prognostic sign for cancer patients; Tregs may play a role in the

suppression of antitumor immunity and lead to poor survival (58, 59). Upon recognition $CD8^+$ cytotoxic T-cells can directly lyse tumour cells via perforin/granzyme or Fas/FasL mechanisms as well as by releasing IFNγ that promote tumour regression. Nevertheless, some $CD8^+$ cytotoxic T-cell subsets secrete IL-10, which is regarded as an anti-inflammatory, immune-suppressive cytokine promoting tumour immune escape (60). Generally, a significant $CD8^+$ T-cell infiltration is associated with a better prognosis for survival (61).

B-Lymphocytes: B-cells can target tumour cells via antibody production as well as by presenting antigen to T-cells (62). However, B-cells obstruct T_H1 activation which activates $CD8^+$ cytotoxic T-cells (63), and B cells have been shown be suppressive in several murine tumour models. Furthermore, the presence of tumour-specific antibodies in the serum of cancer patients, including mesothelioma patients is not indicative of a better prognosis.

Tumour and treatments

Several studies have been conducted to block angiogenesis signalling cascades. ***Vascular targeting therapies include***:

Anti-VEGF Agents: This treatment blocks VEGF from binding to its receptors (VEGFRs) (16); e.g. anti-human VEGF antibodies such as Bevacizumab resulting in reduced endothelial cell proliferation, migration, survival, and permeability (64). When combined with standard chemotherapy Bevacizumab improved overall survival in metastatic colorectal cancer (65), this may be due to improved chemo-perfusion through the tumour bed. Furthermore, breast cancer-bearing mice treated with Bevacizumab lead to modulation of tumour-associated immune cells, where a significant infiltration of TANs into the tumour was reported as well as reduced TAMs numbers. The effect of anti-VEGF antibodies on blood vessels and immune cells has not been studied on mesothelioma and lung cancer.

Anti-VEGF-Receptor Agents: This approach interferes with angiogenesis signalling cascades by blocking the VEGFR- receptors (VEGFR1, VEGFR2, NRP-1, and NRP-2) that are expressed on endothelial cell surfaces. Similarly, sorafenib and sunitinib have been used to target VEGF receptors in endothelial cells and showed a meaningful improvements in patients with gastrointestinal stromal tumours (67). For example, administering a lower-dose of the anti-VEFRR1 antibody, DC101, to breast cancer-bearing mice contributed to improved $CD4^+$ and $CD8^+$ T-cell tumour infiltration (68). Similar to anti-VEGF agents, the effect of anti-VEGFR agents on vessel modulation and immune cell alteration in mesothelioma and lung cancer has not been determined.

Copper Depletion: Copper is essential to pro-angiogenic factors, such as VEGF, that stimulate endothelial cell proliferation and migration (69, 70). A study on a murine mesothelioma model showed that copper depletion contributed to reduced endothelial cell proliferation and activation, narrowed blood vessel diameters, and promoted $CD4^+$ T cell infiltration (70). The effect of copper levels on neutrophils, DCs, and macrophages in MM and lung cancer has not been studied.

Immunotherapies work by inducing or enhancing an anti-tumour immune response. The use of immunotherapies to treat MM and lung cancer tumours represents a promising strategy, especially when combined with other treatments. They may be even more effective when combined with vascular targeting therapies that improve their perfusion.

Agonist anti-CD40 antibodies and chemotherapy: CD40 is a co-stimulatory molecule and is a member of the tumour necrosis factor super family. It is expressed on the surface of different cell types, including DCs, macrophages and B-cells as well as on tumour-associated endothelial cells (71-74). CD40L is expressed mostly on activated $CD4^+$ T-cells and

sometimes on activated $CD8^+$ cells (75). Agonist anti-CD40 monoclonal antibodies stimulate the immune response against a tumour by their affinity to CD40, however, the response is rarely enough to eradicate the tumour (76). Chemotherapeutic agents such as doxorubicin and gemcitabine induce tumour cell apoptosis, which provides an abundant source of tumour antigens for DCs (76). Administration of an agonist anti-CD40 antibody after gemcitabine treatment mediated tumour regression in a murine mesothelioma model; activation of $CD8^+$ T-cells was critical in mediating tumour regression in this scenario (77). Inclusion of a vascular targeting agent in this regimen may provide an even better outcome.

Agonist anti-CD40 antibodies and IL-2: A study conducted on a transgenic mouse model of islet cell carcinogenesis showed that anti-CD40 antibody induced distinct changes to tumour endothelial cells including a reduction in tumour blood vessels, diminished tumour blood supply, increased levels of tumour hypoxia and improved immune cell infiltration (73). Inclusion of IL-2 reduced tumour growth and promoted anti-tumour immunity. These data showed that anti-CD40 antibody may function as a vascular targeting agent rather than an immunotherapy. Nonetheless, inclusion of a vascular targeting agent in this regimen may further improve the outcome.

IL-12: IL-12 is a potent inducer of $CD4^+$ and $CD8^+$ T cell differentiation and use of IL-12 in a murine model resulted in eradication of mesothelioma tumours with minimal toxicity (78). A vascular targeting agent may improve IL-12-driven outcomes in advanced tumours.

SIGNIFICANCE

Combinations of immunotherapeutic strategies and anti-angiogenesis treatments to treat tumours such as MM have not been studied, and thus represent a gap in tumour therapeutic strategies. Therefore, this project will examine the effects of combining anti-vascular agents with particular immunotherapies on tumour growth, tumour cells, immune cells, and endothelial cells as well as determining whether they have a direct or indirect impact on modulating tumour blood vessels. This study will lead to (1) a greater understanding of tumour blood vessels as a therapeutic target, (2) insights into new potential combination therapeutic targets for mesothelioma and lung cancer, and (3) a better understanding of the rational development of effective therapies that may be more readily translated to the clinic. In particular, this study will assist in the design of therapies that play two important roles; modulating tumour blood vessels and inducing anti-tumour immunity. The design of new, effective therapies will contribute to improved patient outcomes.

RESEARCH METHODS

Mice: Six- to eight-week-old female C57BL/6J mice from the Animal Resources Centre (Perth, WA) will be maintained under standard animal housing conditions at the Curtin University animal facility. All mice will be used in accordance with institutional guidelines and with the approval of the Curtin University Animal Ethics Committee (AEC). To develop tumour growth, mice will be injected subcutaneously with 5×10^5 tumour cells per site. As per AEC conditions, mice will be sacrificed when tumours reach 100 mm^2 diameter (79).

Cell isolation and culture: AE17 mesothelioma tumour cell lines previously derived from the peritoneal cavities of C57BL/6J mice after injection of asbestos fibres (80) and Lewis Lung (LL) cancer cells will be used for this project. Primary endothelial cells will be sourced from murine lungs, hearts and tumours that are rich with microvascular endothelial cells (81). Macrophages will be isolated from the spleen, bone marrow, lymph nodes, peritoneal cavity and tumours (82). Bone marrow, spleen and peritoneal cavity are sources of primary neutrophils (34, 83) (84). DCs will be isolated from mouse spleens, lymph nodes and tumours (85, 86). B- and T-cells will be derived from mouse spleens and lymph nodes and tumours if

possible (87, 88). The table below defines particular cellular biomarkers that may be used in cell-sorting techniques (89).

Cells	Biomarker definition	Activation/regulation markers	Hypoxic markers
ECs	CD31, gap junction markers	ICAM, VCAM	HIF-1, CA-IX, Glut-1 and -3 (glucose transporter), VEGF and osteopontin
CD4+ T	$CD3^+/CD4^+$	CD25, FoxP3, IL-17, Perforin, IFNg, PD-1, CD27, 4-1BB, CTLA-4,CD154 (gp39), CD134 (OX-40), CD95L	
CD8+ T	$CD3^+/CD8\alpha$		
B cells	$CD3^-$/ $B220^+/CD19^+$	CD86,CD80,CD22,CD83	
Neutrophils	$CD11b^+/Ly6G^+$	CD10	
Macrophage	$CD11b^+/Ly6G^-//F480^+$	CD40, CD80, and CD86 TNF, IL-6, IL-10 or IL-12p40/70	
Myeloid DC	$/CD11c^+/MHC\text{-}II^+/CD11b^+$ IFNγ, IL-12		
Plasmacytoid DC	$CD11c^+$/ $PDCA^+$ IFNα		

General methods

Cell viability and proliferation measurement: 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) is a colorimetric assay that measures cell proliferation and viability. The assay is based on the capacity of active cells to cleave the yellow tetrazolium salt (MTT) into a purple formazan crystal colour (70).

Flow cytometry: This technique will be used to analyse ECs, tumour cells, and immune cells derived from mice and co-cultured in vitro. Assessments include cell counting, sorting, and biomarker detection. Flow cytometry is based on fluorescently labelled monoclonal antibodies that specifically recognise markers (antigens). Data analysis will be done via LSR Fortessa and/or FACS Canto II (Becton Dickinson) using FlowJo software (70, 90).

Confocal microscopy: Confocal microscopy is an optical imaging technique used to study treatment effects on immune cells, ECs, and tumour cells using different fluorescent dyes to label cells (70, 91).

Statistical Analysis: Using GraphPad PRISM, a student's *t*-test and one-way analysis of variance (ANOVA) will be used to determine variances between two or more populations, respectively (70).

Research plan

Each of the research aims will be addressed as follows:

Aim #1: To assess the effect of vascular-targeting agents on lung cancer and malignant mesothelioma tumour growth using mouse models.

In vitro studies: Each type of treatment (anti-VEGF, anti-VEGFR, copper depletion, IL-12, and IL-2/anti-CD40 antibodies, chemotherapy) will be separately applied to tumour cells, endothelial cells, and tumour cells co-cultured with endothelial cells. Tumour cell proliferation will be measured using the MTT assay and Ki67 staining by flow cytometry.

In vivo studies: MM or LL cells will be injected into the left flank of C57B/6 mice (87). Mice will be treated with vascular-targeting agents. Tumour measurements will be taken using microcallipers (70). Tumour blood vessels and tumour infiltrating cells will be assessed using flow cytometry and confocal microscopy.

Aim #2: To determine the immunological and vascular impact of anti-vascular agents when used to treat cancer.

In vitro evaluation of each monotherapy by using flow cytometry and MTT assay:

1: Murine-derived immune cells or endothelial cells will be cultured as monocultures and their responses to the vascular targeting agents

2: To better mimic the tumour microenvironment murine-derived immune cells will be co-cultured with tumour cells as a monolayer and as a spheroid and their responses measured as described above.
3: Each murine-derived immune cell co-cultured with endothelial cells as a monolayer.
4: Each murine-derived immune cell co-cultured with tumour cells and endothelial cells as a monolayer and as a spheroid.
5: Only the regimens showing promise in the in vitro studies will be tested in vivo. Tumour antigen presentation, cytotoxic T cell function, and the role of $CD4^+$ and $CD8^+$ T cells will be studied.
In vivo studies*:* will be performed concurrently with the in-vivo study of aim #1.

Aim #3: To determine the anti-cancer potential of combining vascular-targeting agents with immunotherapy.

Data from Aim #1 and Aim #2 will be used as a guide to determine the most efficient types of anti-vascular agents and immunotherapy that could be combined. As new checkpoint inhibitor molecules become available they may also be included in the study after seeking approval from the AEC. This aim includes two objectives:
1) Assessment of the combined treatments on LL and MM tumour growth using mouse models; where *in-vitro* and *in-vivo* studies will be applied in the same way of aim#1 protocol.
2) Determination of the immunological and vascular impact of combined treatments when used to treat cancer; where *in-vitro* and *in-vivo* studies will be applied in the same way of aim#2 protocol.

ETHICAL ISSUES

Animal ethics approval was obtained from Curtin University's Animal Ethics Committee under number AEC_2013_03. I have completed an animal welfare and ethics course at Curtin University. Currently, I am engaged in training in the animal handling techniques that will be used in this project under supervision of Dr. Delia Nelson and Dr. Connie Jackaman. Mice will be handled humanely and monitored regularly. Tumour growth will be measured regularly to ensure that the tumours do not exceed the maximum allowable size. Mice receiving anti-vascular agents and immunotherapy will be monitored for potential side effects, such as weight loss. Procedures to deal with potential adverse events were also established. Where appropriate, advice will be sought from a veterinarian and the animal facility manager.

FACILITIES AND RESOURCES

Most of the facilities and resources that are required for this project, such as the office space, lockable storage space and access to a computer, telephone and printing facilities, will be provided by the School of Biomedical Sciences of Curtin University. The University will also offer lab space and lab equipment, such as such as flow cytometers, confocal microscope, fluorescent microscope, tissue culture hoods, centrifuges, plate readers, cryostat, fridges and freezers for sample storage, which will be used as experimental or data analysis tools. Facilities that are related to the mice and the animal house will also be provided by the University.

DATA STORAGE

The School of Biomedical Sciences of Curtin University will provide the researcher with numbered laboratory notebooks for the recording of experimental data, and these notebooks will be securely stored in the school for a minimum of five years after thesis publication. Electronic data files will be periodically saved on hard drives and DVDs, and copies will be

kept in a secure storage by the researcher, supervisors and the School of Biomedical Sciences for a minimum of five years.

TIME LINE

	Research Task	2014	2015	2016	2017
1	Candidacy proposal	May-Nov			
2	Reading and literature review	X	X	X	X
3	Aim #1 Study	X	X	X	
4	Aim #2 Study	X	X	X	
5	Aim #3 Study		X	X	
6	Data analysis		X	X	X
7	Writing up results for publication			X	X
8	Thesis preparation and submission				X

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