IDENTIFICATION OF BIOMARKERS OF TUMOUR BLOOD VESSELS FOR THERAPEUTIC TARGETING

BY

PHUMLANI TETYANA

A thesis submitted in partial fulfilment of the requirements for the degree of

MAGISTER SCIENTIAE (PHYSIOLOGY)

In the

Department of Anatomy and Physiology
Faculty of Veterinary Sciences, University of Pretoria
South Africa

Supervisor: Dr. Peace Mabeta
Co-supervisor: Dr. Amanda Skepu

FEBRUARY 2014

Summary

Cancer is a disease that develops as a result of unregulated cell growth, whereby damaged cells grow and divide uncontrollably forming lumps of tissue called tumours. Tumours can either be benign, wherein the tumour is confined within a single cluster and cannot spread to neighbouring tissues, or malignant wherein the tumour is mixed with normal cells and has the ability to invade and grow in normal tissues at a different site (Plank and Sleeman, 2003). The growth and development of tumours is dependent on a continuous supply of blood to ensure a sufficient stream of nutrients and oxygen to the tumour and also the removal of metabolic waste products (Neal and Berry, 2006; Neufeld and Kessler, 2006; DuBois and Demetri, 2007). This is achieved through a process called angiogenesis, the growth of a new network of blood vessels from pre-existing vessels (Bouis *et al.*, 2006; Bhat and Singh, 2008).

The aim of the study was to investigate the expression of biomarkers in vascular tumour endothelial (endothelioma) cells and to correlate the biomarker expression with endothelial cell growth and migration, important processes in angiogenesis and tumour progression.

Molecules which interfere with cell function, LY294,002 and PF573,228 were employed in this study. LY294,002 is an inhibitor of Phosphatidylinositol 3-kinase (PI3K) signalling, which is involved in the regulation of cell proliferation, cell growth (size) and survival. PF573,228 is an inhibitor of focal adhesion kinase (FAK). FAK forms a scaffold for the attachment of cells at the extracellular matrix and is an important mediator of cell migration and invasion.

The MTT assay was used to determine the viability of the cells after treatment with the drugs. Real time cell analysis was also used to determine the migration and invasion of endothelioma cells in response to basic fibroblast growth factor, a proangiogenic factor. Light and electron microscopy were employed to investigate morphological changes of the cells following treatment, while caspase activity was measured using an enzyme linked immunosorbent assay (ELISA). Array studies were undertaken to investigate the expression of angiogenic markers in control and drug-treated cells.

A dose-dependent decrease in cell viability and migration were observed in treated cells, with LY294,002 showing a higher potency compared to PF573,228 in both instances. Further studies were undertaken using LY294,002 due to its potency. Light microscopy studies showed that treated cells displayed signs of apoptosis such as an irregular shape and chromatin condensation attributes which were not observed in non-treated cells. This was confirmed by electron microscopy which further revealed signs of apoptosis such as chromatin condensation and margination as well as membrane blebbing. An increase in caspase activity in treated endothelioma cells also confirmed the apoptotic effects of LY294,002.

Alteration of the expression levels of various pro- and anti-angiogenic markers in treated cells was observed. Biomarkers that have been suppressed by LY294,002 in treated cells could offer great insight into cancer progression. Also, these markers can serve as targets for cancer therapy in targeted drug delivery.

Opsomming

Kanker is 'n siekte wat as gevolg van ongereguleerde selgroei ontwikkel. Beskadigde selle groei en verdeel sonder beheer en vorm knoppe (tumours) in weefsels. Tumours kan of goedaardig wees, waar die tumour beperk is in 'n enkele eenheid en verspreiding na naburige weefsel nie plaasvind nie, of kwaadaardig waar kanker en normale selle gemeng is, en die moontlikheid bestaan om normale weefsel in te dring en te groei in 'n ander ligging (Plank en Sleeman, 2004). Groei en ontwikkeling van tumours is afhanklik van 'n onafgebroke bloedtoevoer om voldoende nutriente en suurstof te verskaf en om metaboliese afbreek produkte te verwyder (Neal en Berry, 2006; neufeld en Kessler, 2006; duBois en Demetri, 2007). Dié word bereik deur 'n proses genoem angiogenese, gedefinieer as die groei van 'n nuwe netwerk bloedvate van reeds bestaande bloedvate (Bouis *et al.*, 2006; Bhat en Singh, 2008).

Die doel van die studie was om die voorkoms van biologiese merkers in vaskulêre tumour endoteelselle (“endothelioma” selle) te ondersoek en dit te korrelleer met endoteel selgroei en migrasie, belangrike prosesse in angiogenese en tumour ontwikkeling. Molekules wat selfunksie hinder, LY294,002 en PF573,228 was in hierdie studie gebruik. LY294,002 inhibeer die fosfatidielinositol 3-kinase (PI3K) seinstelsel, wat betrokke is in die regulering van selproliferasie, selgroei (grote) en oorlewing. PF573,228 is 'n inhibeerder van fokale adhesie kinase (FAK). FAK vorm 'n raam vir die aanhegging van selle by die ekstrasellulêre matriks en is 'n belangrike bemiddellaar van selmigrasie en indringing.

Die "MTT" toets was gebruik om die prolifererasie van selle na behandeling met sekere middels te bepaal. "Real time" sel annaliese was ook gebruik om die migrasie en indringing van endoteelselle in response tot basiese fibroblaste groei faktor, 'n proangiogeniese faktor, te bepaal. Lig en elektron mikroskopie was gebruik om die morfologiese sel veranderinge na behandeling te ondersoek. Kaspase aktiwiteit was gemeet deur gebruik te maak van ELISA ("enzyme linked immuno assay") "Array" studies was gebruik om die voorkoms van angiogeniese merkers in kontrole en behandelde selle te bepaal.

'n Dosis afhanklike afname in sel proliferasie en migrasie was opgemerk in behandelde selle, met 'n meer merkbare effek van LY294,002, in dié opsig, in vergelyking met PF573,228 in albei

gevalle. Met hierdie abservasie in gedagte was verdere studies beperk tot LY294,002. Ligte mikroskoop studies het bewys dat behandelde endoteelselle tekens van apoptose getoon het bv. oneweredige vorm en kromatien kondensering, eienskappe wat nie in die kontrole selle waargeneem was nie. Dié is bevestig deur middel van elektron mikroskopie waar tekens van apoptose soos kromatien kondensie, marginasie en membraan "blebbing" gesien was. Verdere bevestiging van die apoptotiese vermoë van LY294,002 was die verhoging in kaspase aktiwiteit in behandelde endoteelselle.

Veranderings in die voorkomsvlakke van verskeie pro- en anti-angiogeniese merkers vir behandelde selle was waargeneem. Biologiese merkers wat deur LY294,002 onderdruk is in behandelde selle kan groter insig gee in die aard van kanker ontwikkeling. Hierdie merkers kan ook as teikens dien vir kankerterapie in geteikende middellewering.

Acknowledgements

I would like to express my sincere gratitude to the following people and institutions for their contribution to the success of the work:

- Dr Peace Mabeta, my supervisor, for her unwavering assistance, tremendous mentorship and professional guidance.
- Dr Amanda Skepu, my co-supervisor, for her continuous support and insightful comments.
- Ms Sannah Nkadimeng for her assistance in the lab, and always making sure that all is in order before every experiment was conducted.
- Mrs Erna van Wilpe and Mrs Lizette du Plessis for their assistance with EM studies
- The endothelioma cell line was provided by Prof M.S. Pepper (University of Pretoria)
- Mintek, the University of Pretoria, Onderstepoort Biological Products, and the National Research Foundation for financial assistance
- The Department of Anatomy and Physiology and the University of Pretoria for facilities
- Special thanks to my parents, Lovelyn Yolisa Tetyana (mother) and the late Oscar Qamata Tetyana (Father), for all the efforts and sacrifices made in ensuring that I realise my dreams and aspirations, words cannot express how grateful I am.
- My siblings (Thulani, Yanga, Akhona, Dumo, Odwa and Aviwe) and all of my friends for their enduring support and words of encouragement.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

µl	=	Microliters
°C	=	Degrees Centigrade
3'-OH	=	3 Prime Hydroxyl Group
AICD	=	Activation-Induced Cell Death
ATP	=	Adenosine Triphosphate
BM	=	Basement Membrane
CDK	=	Cyclin-Dependent Kinases
CIM	=	Cell Invasion and Migration Assay Plate
CO_2	=	Carbon Dioxide
CSC	=	Cancer Stem-like Cells
CSF	=	Colony Stimulating Factor
Da	=	Daltons
ECM	=	Extracellular Matrix
ECs	=	Endothelial cells
EGF	=	Epidermal growth Factor
FAK	=	Focal Adhesion Kinase
FDA	=	Food and Drug Administration
FGF	=	Fibroblast Growth Factor
GPCR	=	G Protein-Coupled Receptor
HCl	=	Hydrochloric Acid
H & E	=	Haematoxylin and Eosin
HIF	=	Hypoxic Inducible Factor

h	=	Hours
HUVEC	=	Human Umbilical Vein Endothelial Cells
IC_{50}	=	Half Maximal Inhibitory Concentration
IFN	=	Interferon
IL	=	Interleukin
IL-8	=	Interleukin 8
LC3	=	Light Chain 3
LY294,002	=	2-4-morpholinyl-8-phenlchromone
M	=	Molar (Moles per litre)
MAbs	=	Monoclonal Antibodies
MMP	=	Matrix Metalloproteinase
mTOR	=	Mammalian Target of Rapamycin
MTT	=	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
PBS	=	Phosphate Buffered Saline
PDGF	=	Platelet-Derived Growth Factor
PDK1	=	Phosphoinositide-Dependent Kinase-1
PF-573228	=	6-((4-((3-(methanesulfonyl) benzyl) amino)-5-trifluoromethylpyrimidin-2-yl) amino)-3, 4-dihydro-1*H*-quinolin-2-one
PI3K	=	Phosphatidylinositol 3-Kinases
PIP2	=	Phosphatidylinositol (3,4)-Bisphosphate
PIP3	=	Phosphoinositide (3,4,5) Triphosphate
PKB	=	Protein Kinase B
RPM	=	Revolutions per Minute

RTCA DP	=	Real-Time Cell Analyzer Dual Plate Instrument
RTK	=	Receptor Tyrosine Kinase
siRNA	=	Small Interfering Ribonucleic Acid
SMI	=	Small Molecule Inhibitors
T308	=	Threonine 308
TEM	=	Transmission Electron Microscope
TGF	=	Transforming Growth Factor
TNF	=	Tumour Necrosis Factor
TSC1	=	Tuberous Sclerosis 1
TSC2	=	Tuberous Sclerosis 2
TSP	=	Thrombospodin-1
U/mL	=	Units per Millilitres
US FDA	=	United States Food and Drug Administration
VEGF	=	Vascular Endothelial Growth Factor
µg/L	=	Micrograms per Litre
Z-VAD-FMK	=	Carbobenzoxy-Valyl-Alanyl-Aspartyl-[O-Methyl]-Fluoromethylketone

Chapter 1

Literature Review

1.1. Cancer

Cancer represents a group of diseases that develop as a result of unregulated cell growth, whereby damaged cells grow and divide abnormally, forming lumps of tissue called tumours or neoplasms. In most instances, cancerous cells proliferate uncontrollably and do not respond to the regulatory signals that control cell growth and apoptosis in normal cells (Radic *et al.*, 2004). This is usually due to the activation of oncogenes and the deactivation of tumour suppressor genes, which results in aberrant cell cycle progression and blockage of apoptotic pathways (Sarkar *et al.*, 2013).

Tumours can either be benign, or malignant. A benign tumour, usually referred to as a non-cancerous lesion, is a tumour that is restricted to the tissue of its origin and lacks the ability to invade neighbouring tissues or spread to distant parts of the body. Cells that make up benign tumours have a decreased growth rate and usually do not impact negatively on nearby tissues. However, some benign tumours have been observed to be pre-cancerous and have the ability to induce cancer if no treatment is administered (Oliveira *et al.*, 2007). Malignant tumours, mostly referred to as cancerous growths, are characterized by their ability to escape their site of origin and invade neighbouring tissues or spread throughout the body forming secondary tumours. This is usually achieved by the use of the circulatory and lymphatic systems which transport cancer cells to distant parts of the body (Cooper, 2000).

The process by which tumours spread and proliferate to distant parts of the body is called metastasis and is the main reason most cancers are extremely difficult to contain or treat (Cooper, 2000). The ability of tumours to become metastatic is mostly brought about by genetic and cellular modifications. These include the down-regulation of cell adhesion receptors which promote tissue-specific cell-cell attachment and also the up-regulation of receptors involved in the stimulation of cell motility (Sarkar *et al.*, 2013).

1.1.1. Types and Classification of Cancer

There are over a hundred types of cancers that have been recorded, but only a few have been seen to occur frequently. Cancers are grouped into four categories based on their tissue of origin. These include Carcinomas, Sarcomas, Lymphomas and Leukaemias (Cooper, 2000).

- *Carcinomas* are amongst the most common cancers known to affect adult humans. These form as a result of anomalies in epithelial cells which make up epithelial tissues, and as such, carcinomas have been classified as cancers that originate from epithelial tissues. Carcinomas include cancers of the breast, lung, prostate and the colon amongst others (Cooper, 2000).

- *Sarcomas* are cancers that originate from supportive or connective tissues. These include cancers of the muscle, bone, cartilage, and fibrous tissues and are rarely noted in humans (Cooper, 2000).

- *Lymphomas* are cancers of lymphocytes, which originate from organs of the lymphatic system, such as lymph glands (Cooper, 2000).

- *leukaemias* are termed "cancers of the blood" and originate in blood forming tissues. They occur due to irregularities in blood-forming cells, namely leukocytes. These types of cancers are the most common in children, accounting for 30% of cancer infections recorded in children below the age of 15 years (Belson *et al.*, 2007).

1.1.2. Risk Factors and Cancer Progression (Carcinogenesis)

Numerous substances and activities have been listed as having the potential to increase the risk of cancer. Cancer inducing substances are usually termed "carcinogens". Risk factors are grouped into exogenous and endogenous factors. Exogenous risk factors include nutritional habits, socio-economic status, lifestyle, physical agents such as radiation, chemical compounds, and biological agents such as viruses. Endogenous risk factors include inherited genetic defects, age, immune system damage, endocrine balance and physiological conditions (Oliveira *et al.*, 2007).

The process by which cancer develops occurs through multiple steps namely initiation, promotion and progression (Oliveira *et al.*, 2007). Initiation occurs when the genetic material of a normal cell becomes defective. This could occur randomly, but very rare, or could be induced by prolonged exposure to carcinogenic substances, called mutagens (Cooper, 2000). The mutations in the genetic material of the cells result in the activation of proto-oncogenes, which encode proteins responsible for the regulation of cell growth and division. Also, mutations result in the deactivation of tumour suppressor genes. When proto-oncogenes are mutated, they form oncogenes which promote uncontrolled proliferation of cells. Cells that have undergone initiation remain similar to surrounding normal cells in terms of morphology but undergo growth and division more frequently and lack differentiation (Vincent and Gatenby, 2008).

Promotion involves continued exposure of defective cells to substances that are capable of inducing carcinogenesis in initiated cells only. Promoters include substances such as growth hormones, steroids or bile acids. On their own, most of these promoters are not carcinogenic, and can only promote cancer development in initiated cells. The highlight of this step is the increase in the proliferation of the mutated cells, to form more cells of the same genotype, through a process called clonal selection (Radic *et al.*, 2004). An increase in the rate at which the cells divide boosts the likelihood of appearance of errors in DNA replication and mutations. Proliferated cells then form a benign tumour. Unlike initiation, this stage is reversible; the benign tumour is capable of regressing in the absence of continued exposure to promoters (Pitot and Dragan, 1991).

The last step in carcinogenesis is the transformation of the benign tumour into a malignant growth, and is known as progression. In this stage, the regulation of cellular growth is impaired completely therefore allowing cells to grow and divide in an uncontrolled manner. Initiated or mutated cells now have the ability to proliferate rapidly without the aid of a stimulus. The mutated cells undertake biochemical, morphological and metabolic changes due to genetic instability and are now capable of invasion. This stage is irreversible, and the formed tumour is capable of metastasizing (Oliveira *et al.*, 2007). A flow diagram illustrating the steps involved in carcinogenesis is shown in Fig. 1.1.

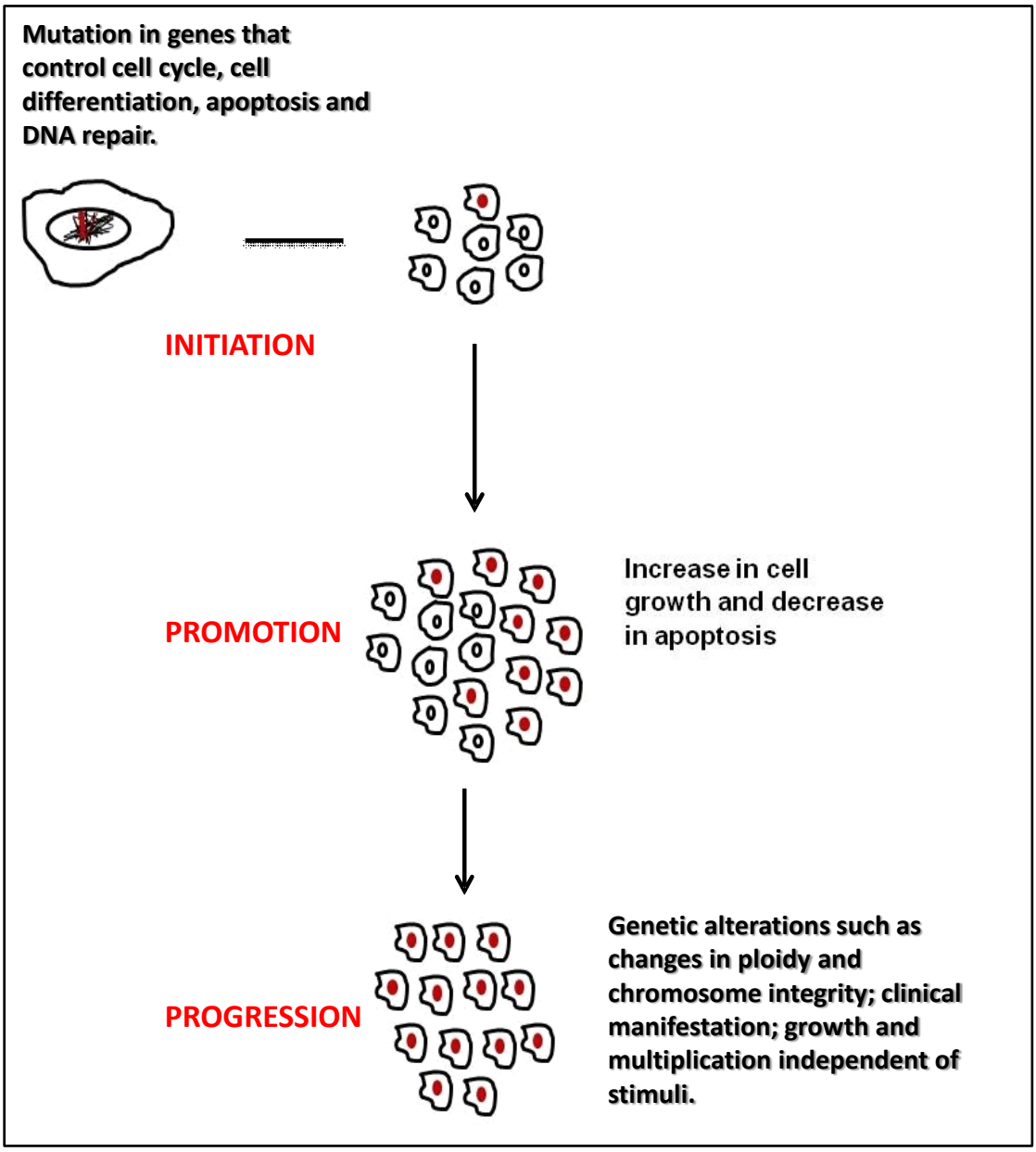

Figure 1.1: Sketch diagram illustrating the steps involved in the process of carcinogenesis. The process begins with the "initiation" step, where a normal cell's genetic material is damaged by a carcinogenic agent like radiation thereby transforming it into a mutated form. When the mutated cell is exposed further to carcinogens, it undergoes the "promotion" step which involves the activation of oncogenes and continued proliferation to form a benign tumour. The mutated cell now enters the last stage, "progression", which involves transformation of the benign tumour into a malignant form (Salvadori and da Silva, 2013).

1.2. Metastasis

Metastasis is the process by which tumour cells migrate from their primary sites to distant tissues, by entering circulation through the blood or lymphatic system (Geiger and Peeper, 2009). This process is the major cause of fatalities in cancer patients, and is the main reason that most cancers are difficult to manage or contain (Fiddler, 2002). More than 90% of deaths in cancer patients are due to the process of metastasis (Valastyan and Weinberg, 2011).

Metastasis is a multi-step process (Fig. 1.2) that involves the following stages: (i) Detachment and migration of tumour cells from the primary tumour. Tumour cells break down the basement membrane (BM), which serves as a barrier between the epithelial tissue and the underlying connective tissue, by producing proteolytic enzymes that compromise the integrity of the BM, (ii) Secretion of growth factors (VEGF, bFGF) by the primary tumour which facilitates the formation of a new vasculature that penetrates the primary tumour, (iii) Migration of cancerous cells (from the primary tumour) to penetrate the newly formed vasculature through a process called intravasation, where they enter the blood or lymphatic system to form clusters called emboli, (iv) Survival of cancerous cells in the harsh conditions of circulation and their effort to circumvent cell death by the process of anoikis, (v) Extravasation of cancerous cells from the blood vessels/circulation to penetrate the tissue in which the blood vessels perfuse, (vi) Continued proliferation of cancerous cells in the new tissue and activation of angiogenesis to form a secondary tumour (van Zijl *et al.*, 2011; Talmadge and Fidler, 2010; Valastyan and Weinberg, 2011). Figure 1.2 illustrates concisely the steps involved in tumour metastasis.

Several molecules have been reported to enhance tumour metastasis. The 37kDa/67 kDa laminin receptor (LRP/LR) is one of the proteins that have been reported to promote metastasis. As such, this protein has been considered as a molecular marker for metastatic spread in various types of cancers (Rea *et al*., 2012). This transmembrane receptor is highly expressed in several types of cancers, where it mediates interactions between tumour cells and the basement membrane. LRP binds laminin-1 with high affinity, leading to increased cell proliferation, cell viability, cell adhesion and cell invasion, important aspects of metastasis (Chetty *et al*., 2014; Omar *et al*., 2012). Laminin is one of the main constituents of the extracellular matrix, which mediates various cellular processes such as movement, extravasation, growth, differentiation, maintenance

of tissue phenotype and survival (Zuber *et al*., 2008). It has been shown that the tumorigenic and metastatic potential of tumour cells increases when they attach to laminin-1 (Omar *et al*., 2012).

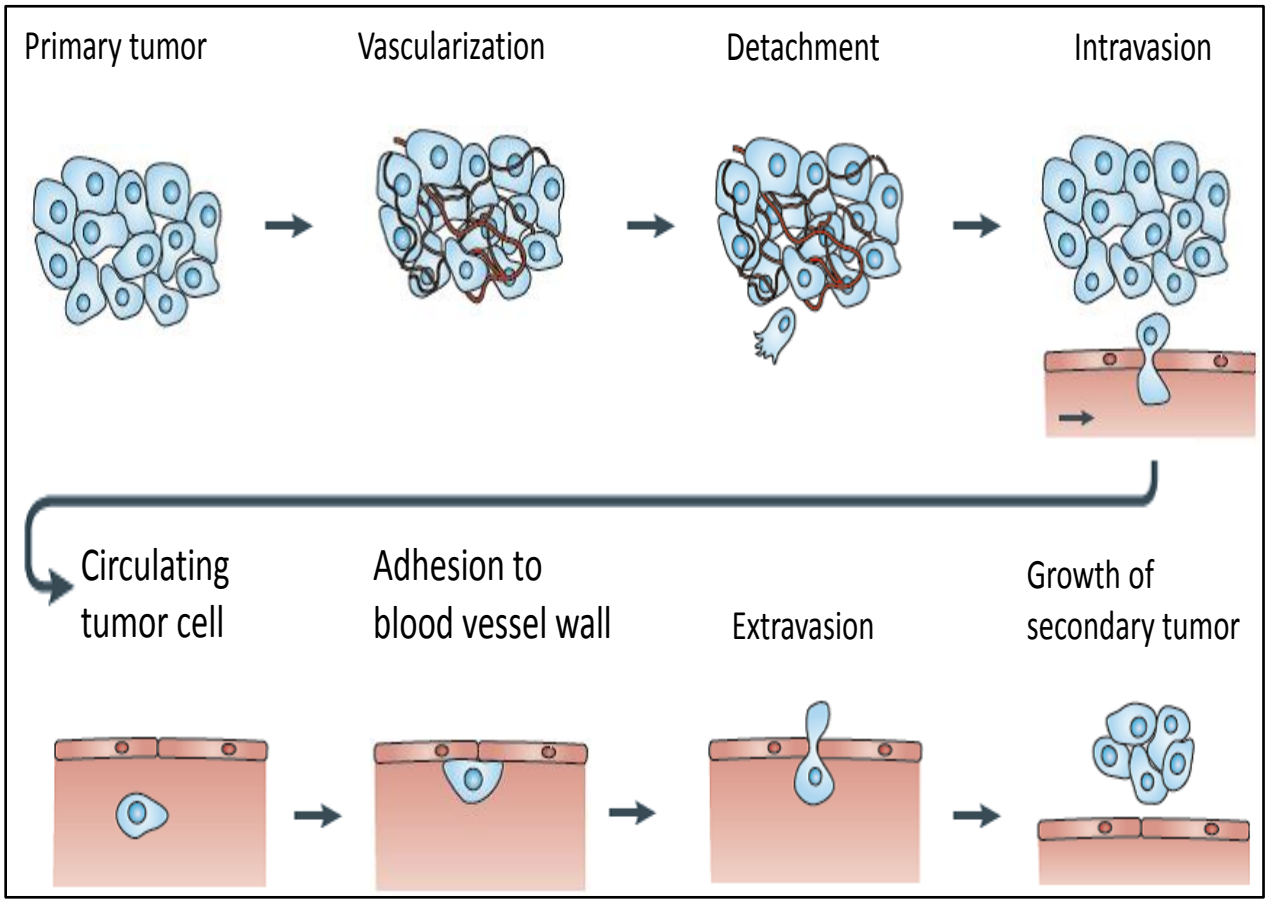

Figure 1.2: Steps involved in tumour metastasis, the process by which cancerous cells migrate from a localised tumour and spread to distant sites of the body where they proliferate to form secondary tumours (Wirtz *et al.*, 2011).

1.3. Pathways Involved in Cancer Development

1.3.1. Phosphatidylinositol 3-kinases (PI3K)/Akt Pathway

Phosphatidylinositol 3-kinases (PI3K) are a group of intracellular signal transducing proteins or enzymes that mediate multiple cellular processes that are critical in the advancement of cancer (Takeda *et al.*, 2010; Courtney *et al.*, 2010). These enzymes act by inducing the formation of phosphoinositide (3,4,5) triphosphate (PIP3) through phosphorylation of the 3'-OH position of the inositol ring of phosphatidylinositol (3,4)-bisphosphate (PIP2) at the cell membrane (Luo *et al.*, 2003; Takeda *et al.*, 2010). Akt is a serine/threonine protein kinase enzyme. It has been implicated in various cellular processes including the regulation of cell survival by blocking pro-apoptotic signals through the phosphorylation of Bcl-2-associated death promoter (BAD), caspase-9 and forkhead transcription factors; regulation of the cell cycle by interacting with cyclin-dependant kinase (CDK) inhibitors such as p21 and p27; regulation of cellular growth by interacting with the tuberous sclerosis 1 and tuberous sclerosis 2 (TSC1/TSC2) complex and mammalian target of rapamycin (mTOR) pathway (Berrie, 2001; Tokunaga *et al.*, 2006).

Phosphatidylinositol 3-kinases and Akt act together in the PI3K/Akt signalling pathway (Fig. 1.3) which is involved in the development of tumours, and has been seen to be highly active in most human cancers. Activation of the PI3k/Akt signalling pathway involves well-orchestrated set of phosphorylation procedures. Initially, PI3K is activated by receptors with protein tyrosine kinase activity (RTK) and G-protein coupled receptors (GPCR), which then produces PIP3 through phosphorylation of PIP2 at the cell membrane (Osaki *et al.*, 2004; Hemmings and Restuccia, 2012). The formed PIP3 binds with Akt thereby allowing phosphoinositide-dependent kinase-1 (PDK1) to activate Akt via phosphorylation of threonine 308 (T308) in the activation loop of the serine/threonine protein kinase (Hemmings and Restuccia, 2012).

The PI3K/Akt pathway has been implicated in the regulation of numerous cellular processes including transcription, translation, proliferation, growth and survival, angiogenesis, metabolism and apoptosis (Osaki *et al.*, 2004; Courtney *et al*., 2010; Wong *et al.*, 2010). Apart from tumour development, PI3K/Akt signalling is also known to influence the tumour response to cancer therapy (Vara *et al.*, 2004). This pathway has been the target in cancer therapy, with numerous

compounds such as LY294,002 halt the spread of cancer through inhibition of PI3K/Akt activity (Vara *et al.*, 2004).

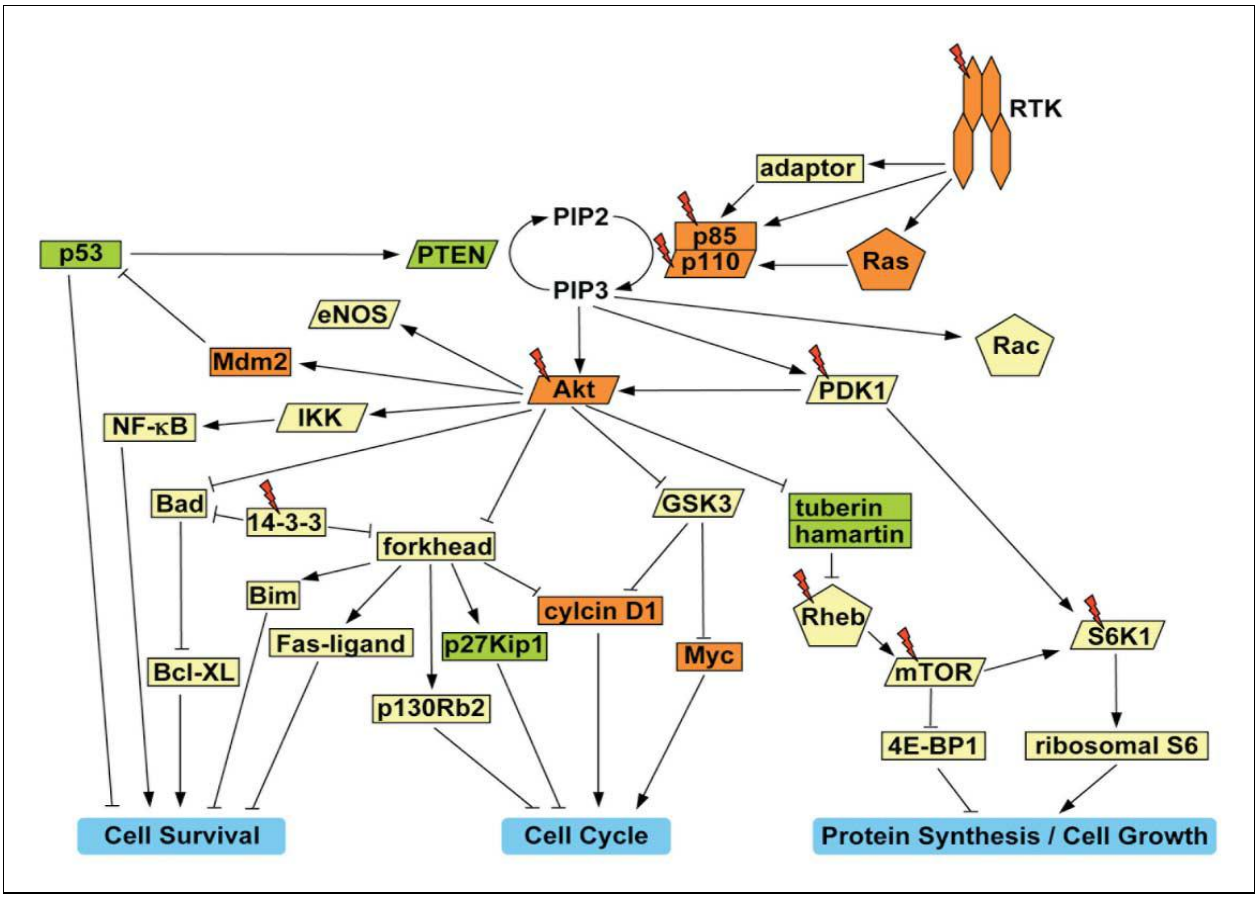

Figure 1.3: An illustration of the PI3K/Akt pathway (Luo *et al.*, 2003).

1.3.2. Focal Adhesion Kinase Pathway

Focal Adhesion Kinase (FAK) is a cytoplasmic, non-receptor protein tyrosine kinase that is frequently produced in various cells (Gan and Siu, 2009). FAK is predominant at the ECM where it forms a scaffold at sites of cell attachment, following activation by the binding of integrins to the ECM or when stimulated by growth factors (Cabrita *et al.*, 2011). It has been listed as one of the protein tyrosine kinases known to regulate integrin and growth factor signalling pathways that mediate biological processes in both normal and cancerous cells (Slack-Davis, 2007). The biological processes include cell growth, cell migration, cell proliferation, and

cell survival. These processes have the ability to induce cancer if they are not carried out properly (Hao *et al.*, 2009). An illustration of the FAK pathway is shown in Fig. 1.4.

An increase in FAK expression in tumour cells has been recorded, with a much higher expression observed in malignant tumour types. A decrease in migration, invasion and proliferation of tumour cells when FAK is inhibited or suppressed has been reported (Parsons, 2003). Furthermore, an increase in the rate of apoptosis and cell detachment was observed when FAK activation was reduced (Hao *et al.*, 2009). With these observations, it is evident that FAK plays a significant role in cancer progression and as such, it has been proposed as a potential target in the fight against cancer metastasis (Hao *et al.*, 2009; Zhao and Guan, 2009). Compounds that have the ability to halt FAK include PF573,228 (section 1.6.2.2).

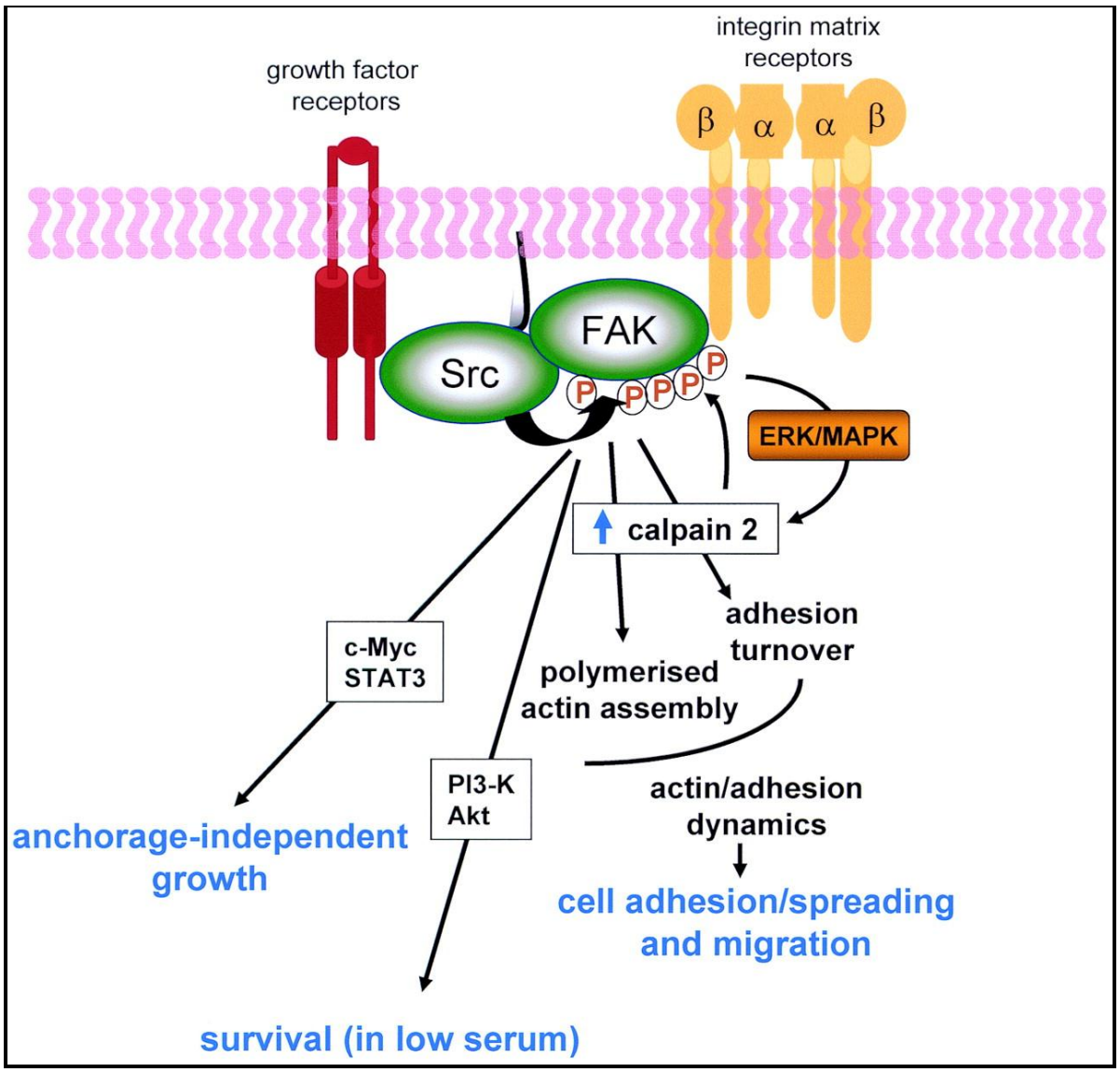

Figure 1.4: Illustration of the Focal Adhesion Kinase pathway (Westhoff *et al*., 2004)

1.4. Angiogenesis

Angiogenesis is the process whereby new blood vessels are formed from pre-existing ones (Makrillia *et al.*, 2008). The process of angiogenesis is seen early in life, where it plays a key role during embryogenesis and foetal development (Tahergorabi and Khazaei, 2012). Also, later in life, angiogenesis remains a critical process. Some of the physiological processes that require angiogenesis in adults include wound healing, follicular development, corpus luteum formation, progesterone release, endometrial growth, regression and repair during the menstrual cycle and development of a fully vascularized tissue for implantation and placentation during pregnancy (Tonini *et al.*, 2003; Tahergorabi and Khazaei, 2012).

Angiogenesis occurs under both physiological and pathological conditions. Under normal physiological conditions angiogenesis is tightly regulated. If angiogenesis occurs excessively, without regulation, it leads to the formation of pathologies such as cancer. Furthermore, insufficient angiogenesis due to poor regulation deteriorates normal physiological processes that require blood vessel formation such as wound healing (Moses, 1997).

1.4.1. Physiological Angiogenesis

Physiological angiogenesis occurs via the sprouting and non-sprouting methods. In the embryo, the primary capillary plexus formed by angioblasts during vasculogenesis is remodelled through angiogenesis to form new micro-vessels (Burrell and Zadeh, 2012).

Physiological angiogenesis plays out in an orderly manner, as shown in Fig. 1.5. Following stimulation, mural cells known as pericytes detach from the sites where the branch is to form on the vessel, in response to the angiogenic stimuli. This is then followed by the degradation and re-modelling of the endothelial cell BM and extracellular matrix (EM) by proteases. Stromal cells form a new matrix which, in conjunction with soluble growth factors, facilitates the growth and spread of endothelial cells (Papetti and Herman, 2002). These endothelial cells form cord-like structures that resemble vessels. Pericytes are then recruited back to the cord-like structures to form a new intact vessel (Nussenbaum and Herman, 2010; Papetti and Herman, 2002).

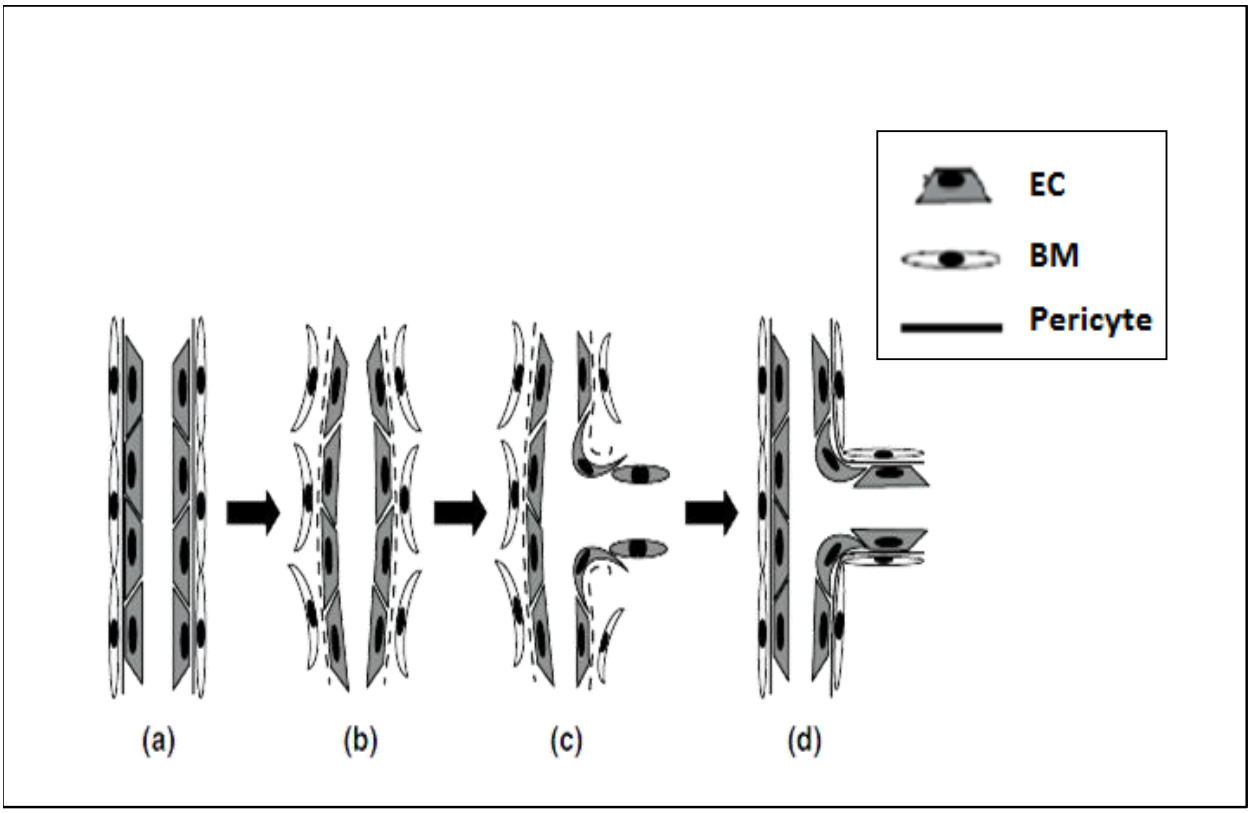

Figure 1.5: Sketch diagram depicting the stepwise process of physiological angiogenesis. a) Normal quiescent vessel with endothelial cells, pericytes and basal lamina in place; b) in the presence of an angiogenic stimuli pericytes detach, the vessel dilates and the basal lamina is degraded; c) ECs proliferate and migrate towards the stimuli; d) ECs form a lumen, pericytes are attached back and the BM is re-established (Liao and Johnson, 2007).

1.4.2. Pathological Angiogenesis

Pathological or tumour angiogenesis is defined as the formation of a new network of blood vessels that penetrates a cancerous growth (Gupta and Qin, 2003). This process plays a primary role during tumour metastasis. The newly formed vasculature serves as a route by which tumour cells escape the site of origin and enter the circulation, thus migrating to distant tissues of the body where they proliferate, forming secondary tumours (Zetter, 1998). In the absence of pathological angiogenesis, tumours remain dormant and unable to grow beyond the size of 1-2 mm (Folkman, 2003). This is attributed to the lack of a continuous supply of oxygen and

nutrients to support tumour growth, and also the accumulation of waste metabolites produced by the tumour (Harlozinska, 2005; Eichhorn *et al.*, 2007).

Tumour angiogenesis follows the same mechanism as normal angiogenesis, with the following steps involved; ECM and BM breakdown, EC proliferation and migration, then arrangement of ECs into stable vessels. The newly formed vasculature is created in such a way that it penetrates the tumour to ensure maximum supply of blood directly to the tumour. The process is initiated by Vascular Endothelial Growth Factor (VEGF) which is secreted by the tumour in response to hypoxic conditions, in a similar fashion as that of normal angiogenesis. The tumour environment becomes hypoxic as the tumour grows beyond 1-2 mm, thereby increasing the demand of oxygen (Nussenbaum and Herman, 2010). Tumour angiogenesis proceeds uninterrupted at such conditions since the regulatory mechanisms that inhibit or switch-off angiogenesis in healthy tissues are impaired, due to an increase in the balance of angiogenesis activators relative to inhibitors (Terman and Stoletov, 2001).

Differences in tumour and normal angiogenesis lie on the morphology and functional attributes of the newly formed blood vessels. Tumour blood vessels are highly disorganized and irregularly distributed as compared to normal blood vessels. Moreover, tumour blood vessels consist of randomly-branched tubular structures with varying flow patterns (Ziyad and Iruela-Arispe, 2011). These vessels show decreased permeability, with vessel walls comprised of ECs and tumour cells (Hashizume *et al*., 2000). The BM is incomplete due to the absence of pericytes on the surface of the vessels causing them to leak or become hyper-permeable (Terman and Stoletov, 2001; Tonini *et al.*, 2003). Lastly, the flow of blood within tumour blood vessels is bidirectional, and perfusion is not sustained (Ziyad and Iruela-Arispe, 2011).

1.4.3. Mechanism of Tumour Angiogenesis

The activation of angiogenesis to support tumour growth and progression has been linked to a phenomenon known as the angiogenic switch (Cavallaro and Christofori, 2000). This is defined as the balance between pro- and anti-angiogenic growth factors or cytokines that regulate the process of angiogenesis. Under normal conditions, the balance of anti-angiogenic growth factors outweighs that of pro-angiogenic factors resulting in the inhibition of angiogenesis. This ensures that angiogenesis will only be activated when new blood vessels are required, and will occur by

altering the balance between activators and inhibitors to favour blood vessel formation (Johnson and Wilgus, 2012).

Thus a tumour may remain dormant for a number of months to years, until it is able to switch to an angiogenic phenotype (Liekens *et al.*, 2001). During dormancy, the rate at which the tumour cells proliferate is proportional to the rate of apoptosis, thereby keeping the tumour under control (Ribatti *et al.*, 2007). The tumour is able to attain an angiogenic phenotype when the pro-angiogenic factors surpass the effects of the anti-angiogenic modulators in the tumour microenvironment, thereby allowing the tumour to evolve into a metastatic form. Various stimuli are involved in the induction of the angiogenic switch including an increase in protein expression activated by oncogenes such as v-*ras*, k-*ras*, v-*raf*, *src*, *fos* and v-*yest*, and various changes in the tumour environment such as hypoxia, depletion of nutrients, and decreased pH (Eichhorn *et al.*, 2007; Semenza, 2000).

The most likely basis for tumours to escape dormancy is hypoxia (Fig 1.6), which is caused by various factors including an increase in the metabolic activity of the tumour, increase in oxygen uptake due to an increase in size of the tumour caused by cell proliferation, and the increase in the distance between the tumour and local capillaries which negatively impacts diffusion of oxygen from the capillaries to the tumour microenvironment (Liao and Johnson, 2007). Under hypoxic conditions, tumours produce a transcriptional factor known as hypoxia inducible factor (HIF)-1 which induces the expression of one or more pro-angiogenic factors such as Vascular Endothelial Growth Factor (VEGF), Fibroblast Growth Factor (FGF-2), Interleukin-8 (IL-8), Transforming Growth Factor (TGF-b), Platelet Derived Growth Factor (PDGF), pleiotrophins. Apart from hypoxia, HIF can also be induced in conditions of genetic modification by tumour cells, as shown in Fig. 1.6 (Semenza, 2000).

HIF-1 is a heterodimeric transcriptional protein, containing HIF-1α and HIF-1β subunits. HIF-1α is a cytoplasmic protein that is regulated by oxygen levels in the tumour microenvironment and HIF-1β is a nuclear protein that is not affected by oxygen levels. In the presence of adequate oxygen concentrations, HIF-1α is deactivated, owing to increased activity of the ubiquitin-proteasome system that degrades HIF-1α. In conditions of hypoxia, where oxygen is limited, HIF-1α is activated and translocated to the nucleus where it binds to HIF-1β to form the

heterodimer HIF-1. The heterodimer then binds to DNA (in specific hypoxic response elements) and activates the transcription of genes that code for products essential for tumour angiogenesis (such as VEGF) among others (Vaupel, 2004; Ravi *et al.*, 2000).

The most potent inducer of angiogenesis in such conditions is VEGF, whose expression is further augmented by the production of other growth factors. This is accompanied by down-regulation of the expression of molecules that negatively modulate angiogenesis such as the tumour suppressor gene, p53, which produces and activates Thrombospodin-1 (TSP-1), which negatively regulates angiogenesis (Eichhorn *et al.*, 2007; Ribatti *et al.*, 2007; Sugimachi *et al.*, 2002; Liekens *et al.*, 2001). The secreted pro-angiogenic factors, either by tumour cells or by host cells, induce the angiogenic switch by altering the functional state of endothelial cells on pre-existing vessels, after which tumour angiogenesis occurs (Francavilla *et al.*, 2009).

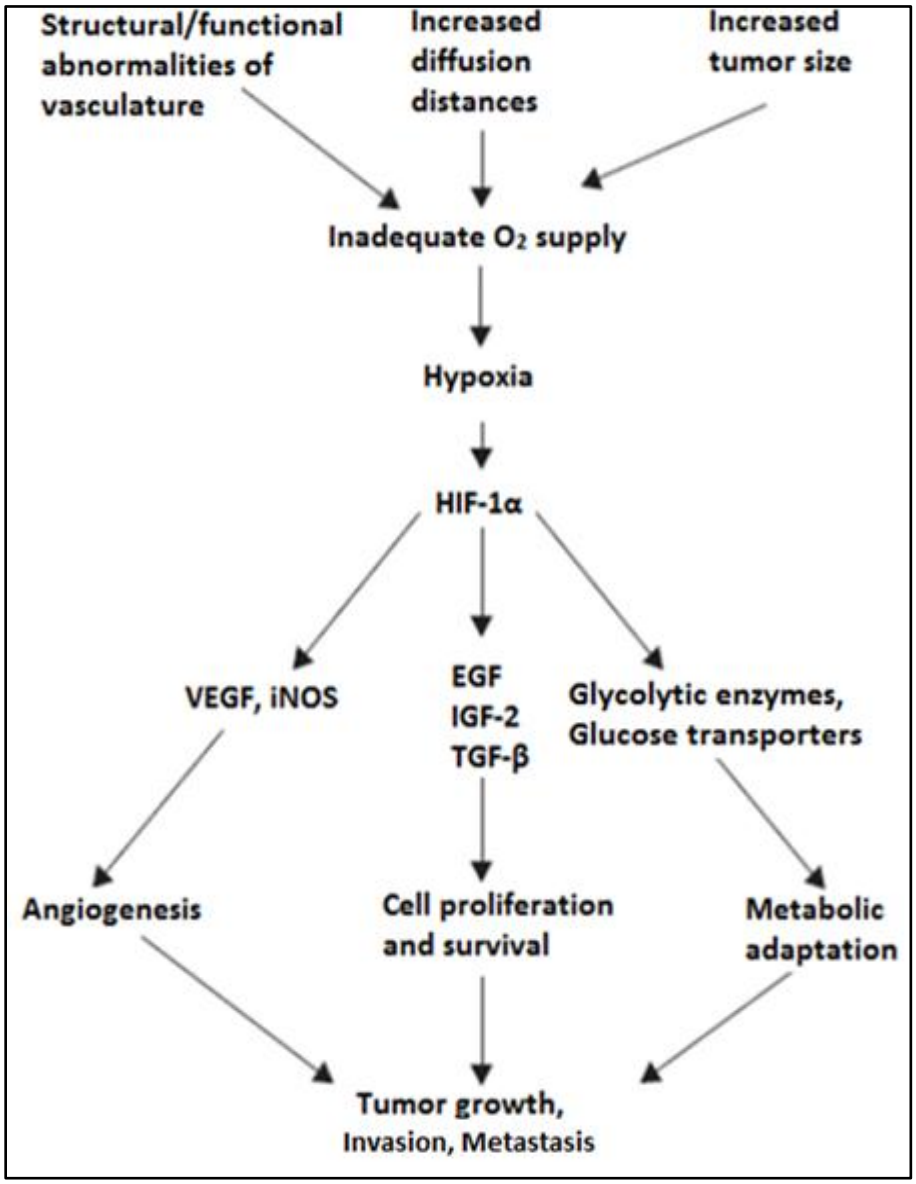

Figure 1.6: Role of HIF-1α in the initiation of angiogenesis. As the tumour grows in size, its metabolic activity increases resulting in rapid O_2 consumption, leading to hypoxia. Under hypoxic conditions, the tumour initiates production of the HIF-1 transcription factor which in turn induces the production of pro-angiogenic growth factors such as Epidermal growth factor (EGF), VEGF and TGF which leads to the initiation of angiogenesis and other angiogenesis related processes, which support tumour growth (Vaupel, 2004).

1.4.4. Regulation of Angiogenesis

The regulation of angiogenesis is mediated by various molecules including growth factors and cytokines. Uncontrolled secretion of these cytokines and growth factors induces uncontrolled endothelial cell proliferation observed in tumour neovascularisation and in angiogenesis dependant diseases (Presta *et al.*, 2005).

1.4.5. The Role of Cytokines in Angiogenesis

Cytokines are a group of small cell signalling, soluble glycoproteins and low molecular weight peptides**.** These are intracellular communicating molecules involved in immunity, inflammation and repair, and general tissue homeostasis. Furthermore, cytokines can facilitate the production of other cytokine members which elicit malignancy from normal cells such as the tumour-associated macrophages (Dunlop and Campbell, 2000). Cytokines include members of the Tumour necrosis factor (TNF), Interleukin (IL), Interferon (IFN), Colony-stimulating factor (CSF) families and many more (Wilson and Balkwil, 2002).

The laminin receptor protein (LRP/LR) also plays a major role in the regulation of angiogenesis. As previously explained, LRP/LR is a high affinity receptor for laminin. Laminins are trimeric glycoproteins that mediate endothelial cell adhesion, differentiation and tube formation. Also, laminins regulate the activity of endostatin, which is an angiogenesis inhibitor that is known to inhibit tube formation (Khusal *et al*., 2013). When bound to laminin-1, LRP/RP activates various physiological processes that are important in the process of angiogenesis. These include cell growth, cell migration and cell adhesion (Khumalo *et al*., 2013; Givant-Horwitz *et al*., 2005).

1.4.6. The Role of Growth Factors and Their Receptors

Growth factors are grouped into two classes; those that have a direct effect on endothelial cells and those that act on endothelial cells through the release of other growth factors (Mousa, 2008). The most commonly studied growth factors include the VEG and FGF. When secreted, VEGF and FGF stimulate the production of proteases and plasminogen activators which lead to the degradation of the vessel BM causing cells to migrate into the surrounding matrix (Cross and Cleasson-Welsh, 2001).

Vascular Endothelial Growth Factor family comprises of three specific cell surface receptors, namely VEGFR-1, VEGFR-2, and VEGFR-3 (Cross and Cleasson-Welsh, 2001; Bouis *et al.*, 2006). VEGF is the best characterised growth factor that plays a unique role in the regulation of physiological and pathological angiogenesis. Vascular Endothelial Growth Factor is highly expressed in several types of tumours (Gupta and Qin, 2003). This growth factor is involved in key activities of endothelial cells such as secretion of proteases, migration and proliferation, and is also involved in the inhibition of apoptosis thereby prolonging survival (Cross and Cleasson-Welsh, 2001; Bouis *et al.*, 2006).

Vascular Endothelial Growth Factor enhances endothelial cell permeability by loosening adhering junctions between cells in a monolayer by rearranging the cadherin/catenin complexes leading to vessel leakage (Gupta and Qin, 2003). Vascular Endothelial Growth Factor is also capable of up-regulating cell-substrate adhesion molecules causing a shift in the adhesive balance from cell-cell adhesion towards cell-matrix adhesion (Plank and Sleeman, 2003).

Fibroblast Growth Factors belongs to the family of heparin-binding growth factors that are strong mitogens of various cell types. The growth factors elicit various complex and well-coordinated processes such as cell proliferation and survival, chemotaxis and protease production in cultured endothelial. Without FGFs, human ECs fail to proliferate and therefore undergo apoptosis *in vitro* (Mousa, 2000). This family contains 9 members and only two are well characterised as mediators in angiogenesis, namely acidic FGF (aFGF or FGF1) and basic FGF (bFGF or FGF2) (Bouis *et al.*, 2006).

Similar to VEGF, FGFs are highly expressed in cancers where they orchestrate various biological processes. These include the activation of endothelial cell proliferation and migration, and promoting micro-vessel tube formation (Nussenbaum and Herman, 2010). Also, bFGF has been reported to promote survival of endothelial cells by activation of anti-apoptotic proteins such as bclXL and bcl-2 through the MEK/ERK pathways (Gupta and Qin, 2003). Fibroblast Growth Factors also play an important role in blood vessel remodelling in wound healing and also in tumor angiogenesis (Nussenbaum and Herman, 2010).

1.4.7. Apoptosis Inhibition during Angiogenesis

The inhibition of apoptosis is important in the process of angiogenesis (Mabeta and Pepper, 2009). Inhibition of apoptosis helps promote endothelial cell survival, an important requirement for angiogenesis. Angiogenesis inhibitors, such as endostatin, target endothelial cells where they induce apoptosis (Mabeta and Pepper, 2009). Growth factors, particularly VEGF and bFGF, inhibit endothelial cell apoptosis thereby inducing angiogenesis (Chavakis and Dimmeler, 2002). Both VEGF and bFGF have been shown to induce endothelial cells to form capillary-like tubes *in-vitro* (Mabeta and Pepper, 2009). Figure 1.7 illustrates concisely the mechanisms by which VEGF and bFGF inhibit apoptosis.

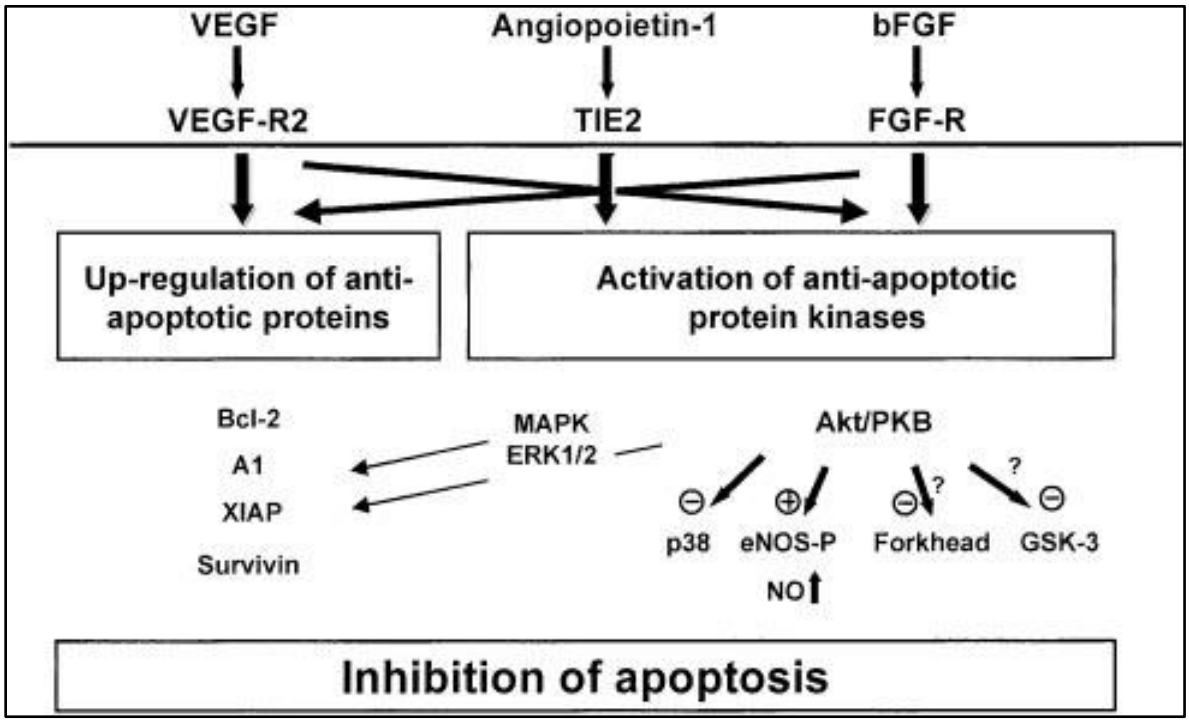

Figure 1.7: Inhibition of apoptosis by angiogenesis growth factors to promote angiogenesis (Chavakis and Dimmeler, 2002).

Receptors, such as LRP/RP, have also been reported to promote angiogenesis by inhibiting apoptosis to maintain cell viability. Susantad and Smith (2008) reported an increase in the rate of apoptosis in transformed liver cells when LRP/RP was knocked out. This was further confirmed by Moodley and Weiss (2013), when they recorded a decrease in the viability of A549 and Hela cells, when LRP/LR was down regulated using siRNA.

1.5. Anti-angiogenic Agents in Cancer Treatment

In the past, various treatment methods were employed to treat cancer, with surgery, radiation, and cytotoxic chemotherapy amongst the most used modalities (Baillie *et al.*, 1995; Gerber, 2008). Chemotherapy remains the therapeutic modality of choice although numerous serious side effects have been associated with its use. For instance, chemotherapeutic drugs kill rapidly dividing cells. These drugs kill malignant cells and proliferating normal cells, hence patients that undergo this kind of treatment suffer from ailments such as hair-loss and vomiting (Vapiwala and Geiger, 2010). Furthermore, chemotherapeutic drugs depend on the vascular system to reach the site of the tumour, and in most cases tumours lack proper perfusion, thereby limiting access of the drug to the tumour site (Baillie *et al.*, 1995; Gerber, 2008).

Anti-angiogenic drugs inhibit the process of angiogenesis and are therefore important in cancer therapy. These drugs normalise tumour blood vessels thus ensuring that anti-cancer drugs are able to reach tumour cells. Most of these anti-angiogenic drugs target the VEGF pathway, which is amongst the most specific and critical regulators of angiogenesis (Pandya et al., 2006). However, angiogenesis can occur through alternative pathways, and therefore tumours can employ such pathways to avoid anti-VEGF therapy (Sagar *et al.*, 2006). This raises the need to target angiogenesis using alternative pathways.

1.6. Targeted Therapy

In the search for alternative treatment routes, targeted therapy was proposed. This involves the use of agents that specifically target molecules or pathways involved in cancer development and progression (Gerber, 2008). Targeted pathways include tumour angiogenesis, apoptosis, regulation of gene transcription and signal transduction (Xue *et al.*, 2012). Target molecules are usually those that are ubiquitously expressed in normal tissue, but are found to be defective and

overexpressed in tumours (Gerber, 2008). Some of the molecules that are usually targeted include molecules that regulate angiogenesis, molecules that facilitate cell invasion, modulators of apoptosis, growth factors and their receptors, and cell-cycle proteins (Widakowich *et al.*, 2007).

Targeted therapy offers vast advantages over conventional therapeutic modalities. Firstly, drugs used in targeted therapy have a high therapeutic index, since they specifically target a unique feature available in tumour cells only. Secondly, since the target molecule is critical in the formation of the cancer, it is less likely to develop resistance to the drug (Xue *et al.*, 2012). Currently, targeted therapy mainly involves the use of two types of agents; monoclonal antibodies and small molecule inhibitors (Gerber, 2008).

1.6.1. Monoclonal Antibodies

The use of monoclonal antibodies (MAbs) in cancer therapy has been effective for over 15 years, and is amongst the leading modes of treatment for haematological malignancies and solid tumours (Scott *et al.*, 2012). Their inherent ability to specifically bind cancerous cells with high affinity and elicit anti-tumour activity has made MAbs one of the major therapeutic modes in oncology (Ross *et al.*, 2003; Ross *et al.*, 2004; Zhang *et al.*, 2007). Since MAbs are large in terms of their size (150 000 Da), they cannot easily penetrate the plasma membrane of cells. Therefore monoclonal antibodies are usually designed to target substances on cell surfaces or outside the cells, such as extracellular components of pathways, ligands and receptor binding domains (Gerber, 2008; Xue *et al.*, 2012).

Various methods by which MAbs elicit anti-tumour activity have been postulated. Firstly, MAbs can trigger an attack on the target cell or molecule or label the target molecule for destruction by host immune defences. Secondly, MAbs have the ability to bind to ligands or receptors on target cells and halt or destruct processes that promote carcinogenesis. Lastly, MAbs can induce anti-tumour activity by directing a certain anti-cancer agent to the target cell. This applies to monoclonal antibodies that have been conjugated to anti-cancer substances such as radioisotopes (Gerber, 2008). Thus far, 12 MAbs have been approved by the Food and Drug Administration (FDA) for use in patients with haematological malignancies and solid tumours, and many more are currently undergoing clinical trials (Scott *et al.*, 2012).

1.6.2. Polyclonal Antibodies

Polyclonal antibodies (PAbs) have also been used to inhibit several pathways that lead to cancer progression, although these haven't been used as much as MAbs. Polyclonal antibodies such as the anti-LRP/LR specific antibodies, W3 and IgG1-iS18, have been shown to exhibit anti-cancer properties. Both antibodies have been reported to block angiogenesis and tumour metastasis *in vitro*. W3 was reported by Khusal and colleagues (2013) to inhibit the formation of tubular structures in HUVEC angiogenesis models, signifying its potential as a tool for therapeutic targeting. Also, IgG1-iS18 was reported by Chetty *et al* (2014) to inhibit the interaction between laminin-1 and LRP/RP, thereby reducing adhesion and invasion in liver cancer cells.

1.6.3. Small Molecule Inhibitors

Small molecule inhibitors are organic chemicals, with an average molecular weight of 1000 Da. They can be administered intravenously or orally (Xue *et al.*, 2012). They are less expensive to prepare compared to MAbs since they are chemically synthesized (Gerber, 2008). Small molecule inhibitors (SMIs) are designed to target substances within cells since they are capable of penetrating the cell membranes (Xue *et al.*, 2012). In most cases, SMIs are intended to halt cancer progression by blocking intracellular signalling of receptor and non-receptor tyrosine kinases such as EGFR and VEGFR (Gerber, 2008). In most cancers, various kinases are usually activated and mutated, making them suitable targets. Small molecule kinase inhibitors block kinase activity through inhibiting cross-phosphorylation of kinase domains and phosphorylation of substrates downstream on the signalling cascade (Chen *et al.*, 2008).

Small molecule kinase inhibitors achieve kinase inhibition using various modes. Firstly, the inhibitor occupies or binds to the ATP binding pocket in the active site of an activated kinase, thereby preventing ATP from binding and initiating a signalling cascade. Secondly, the inhibitor can bind to two different sites on the kinase, that being the ATP binding pocket and another binding site that is only accessible in inactive kinases, thereby ensuring that the kinase remains inactive. The third mode of inhibition involves binding of the inhibitor to sites distant from the ATP binding pocket, such as the substrate binding site, to block interaction of the kinase with its substrate (Chen *et al.*, 2008).

1.6.3.1. LY294,002

LY294,002, also known as 2-4-morpholinyl-8-phenlchromone, is a derivative of a kinase inhibiting bioflavonoid called quercetin (Takac *et al.*, 2013). This synthetic compound is a potent inhibitor of PI3K that binds in a reversible manner to the active site of the catalytic subunit of PI3K (Sun *et al.*, 2004). LY294,002 competes with adenosine triphosphate (ATP) for the active site and is a very potent inhibitor that elicits a response in micro-molar doses (Wymann *et al.*, 2003). It is due to such activity that this compound has been used extensively to investigate the significance of PI3K signalling pathways in cellular responses (Ward *et al.*, 2003).

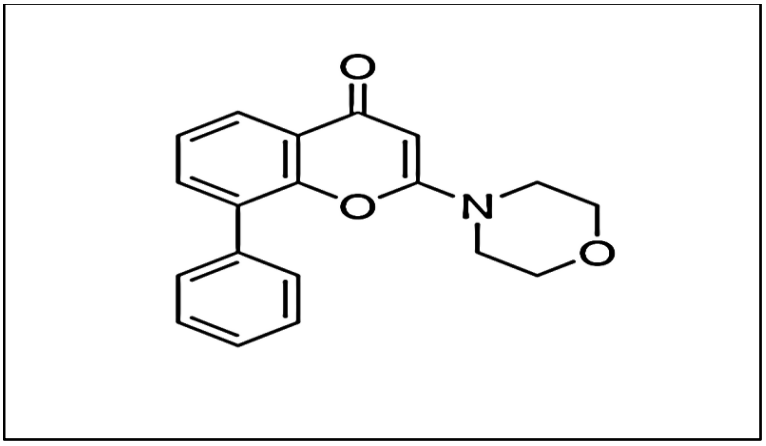

Figure 1.8: Molecular structure of the PI3K inhibitor, LY294,002.

LY294,002 has been observed to elicit anti-proliferative and pro-apoptotic activities in cells (Vara *et al.*, 2004). It stimulates cell cycle arrest during cell growth therefore impeding cell proliferation by blocking hyper-phosphorylation of the retinoblastoma protein and promoting the up-regulation of p27, a cyclin-dependant kinase inhibitor (Hu *et al.*, 2000). Furthermore, LY294,002 has been reported to exhibit high anti-tumour activity (Jiang *et al.*, 2013). Although LY294,002 has shown remarkable potential in combating the detrimental effects of PI3K in cancer progression, its clinical development has not seen much progress due to its pharmacological deficiencies such as limited stability, insolubility and toxicity (McNamara and Degterev, 2011; Stein, 2001).

1.6.3.2. PF573,228

[6-((4-((3-(methanesulfonyl) benzyl) amino)-5-trifluoromethylpyrimidin-2-yl) amino)-3, 4-dihydro-1*H*-quinolin-2-one] (PF573,228) is a potent and selective inhibitor of FAK developed by Pfizer. This is a cell permeable pyrimidinyldiamino compound that interacts with FAK in the ATP-binding pocket and blocks its catalytic activity with a half maximal inhibitory concentration (IC_{50}) of 4 nM (Slack-Davis, 2007). PF573,228 acts by preventing the phosphorylation of cellular FAK on Tyrosine residue 397 and simultaneously blocking phosphorylation of its downstream effector, paxillin, on Tyrosine residue 31 (Hao *et al.*, 2009). This compound is capable of blocking cell migration in cultured cells, but has not managed to inhibit proliferation, cell growth and survival, or induce apoptosis (Zhao and Guan, 2009).

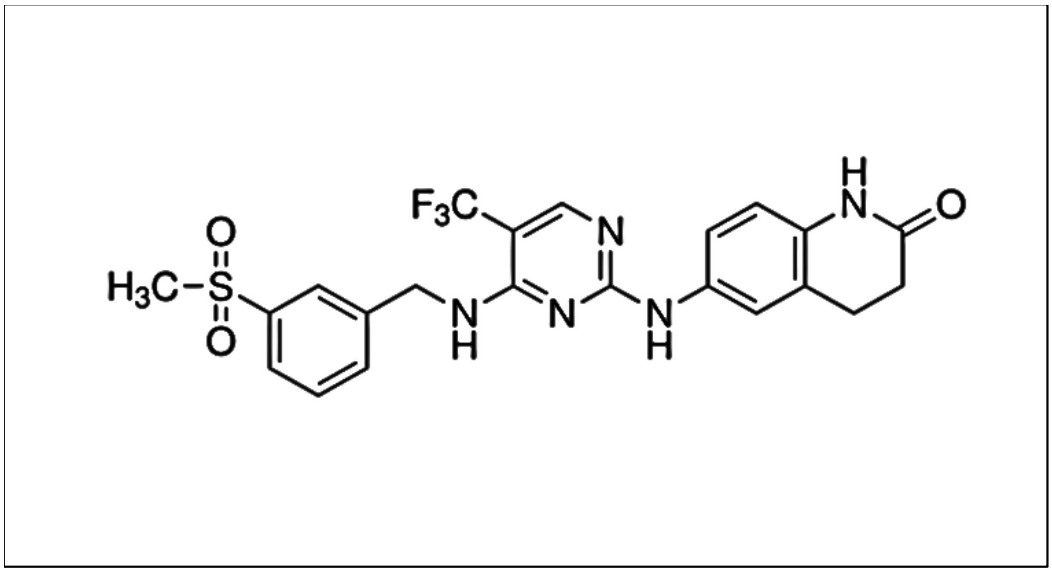

Figure 1.9: Molecular structure of the FAK inhibitor, PF573,228.

1.7. Problem Statement

Cancer is one of the leading causes of death in South Africa, with approximately 42 000 deaths recorded in 2012 alone (Ferlay *et al.*, 2013). Cancer deaths are due to metastasis which is usually driven by angiogenesis. Cancer treatment poses many challenges due to the lack of specificity (drugs target cancer and normal cells), the inability to reach cancerous tumours due to the tortuous nature of tumour blood vessels, as well as the severe toxicity of current treatment methods. There is a need for targeted therapy, especially drugs that are able to inhibit important pathways in tumour development and progression.

1.8. Null Hypothesis

H_0: Angiogenic parameters and the expression of regulatory biomarkers in tumour endothelial cells are not affected by small molecule inhibitors that target PI3k and FAK.

1.9. Aim and objectives

The aim of this study is to determine the effects of small molecule inhibitors, LY294,002 and PF573,228, on aspects of angiogenesis (cell proliferation, migration, and apoptosis inhibition) using endothelioma cells. In addition the study aims to identify angiogenic biomarkers in these tumour endothelial cells, and to investigate any changes in maker expression in response to treatment.

The objectives of the study are:

i. To study the effects of LY294,002 and PF573,228 on endothelioma cell growth and migration, both important aspects of angiogenesis.
ii. To study the morphology of endothelioma cells in response to treatment.
iii. To investigate caspase-3 activity in response to treatment.
iv. To investigate markers expressed by endothelioma cells.
v. To investigate the expression of these markers in response to treatment.

1.10. Benefits Arising From the Project

If effective, small molecule inhibitors with anti-tumour and anti-angiogenic effects will be valuable in the quest to identify effective molecules for the treatment of cancer. Also, the biomarkers identified in this project could be useful as therapeutic targets, diagnostic or prognostic markers.

Chapter 2

Materials and Methods

2.1. Cell Maintenance

Endothelioma cells (obtained from Prof M.S. Pepper, University of Pretoria) were maintained in 25 cm^2 tissue culture flasks (Whitehead Scientific, Johannesburg, South Africa) in a humidified atmosphere containing 5% CO_2 at a temperature of 37°C. Endothelioma cells are endothelial cells derived from tumour blood vessels. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% foetal calf serum (Scientific Group, Midrand, South Africa) and 1% penicillin-streptomycin (Life Technologies, South Africa). Medium was changed every second day. All experiments were repeated in order to validate the results.

2.2. Trypan Blue Exclusion Assay

Trypan blue exclusion assay measures cell viability. Viable cells have intact cell membranes that do not allow trypan blue to go through and therefore appear clear and unstained, whereas non-viable cells have compromised membranes that allow trypan blue to permeate through, and are therefore stained blue (Strober, 2001).

Cells were detached from flasks by use of trypsin-EDTA solution. Cells were centrifuged at 200 x g for 10 min and then re-suspended in medium. An aliquot (15 µl) of the cell suspension was diluted with 5 µl of phosphate buffered saline (PBS), and then mixed with 15 µl of trypan blue in a small tube. A aliquot (15 µl) of the trypan blue - cell mixture was then applied to the haemocytometer counting chamber, which was then placed on a light microscope to count the number of viable cells. The total number of viable cells/ml of aliquot was calculated using the formula:

$$\textit{Viable cells/ml} = \textit{Total number of counted cells} \times 2500 \times \textit{Dilution Factor (DF)}$$

$$\textit{Where DF} = \frac{\textit{Total volume of trypan blue-cell suspension}}{\textit{Aliquot volume}}$$

2.3. Cell Viability Assay

Cell viability was studied using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay according to Riss *et al* (2004). The MTT assay measures the mitochondrial activity of cells through their ability to convert MTT into formazan, with an absorption maximum at 570 nm (Riss *et al*, 2004). MTT is a yellow soluble tetrazolium dye, which when cleaved by mitochondrial enzymes (such as mitochondrial dehydrogenase) forms a blue insoluble precipitate that is deposited inside cells. The amount of formazan formed is directly proportional to the number of viable cells present during exposure to MTT, since only viable cells are capable of catalysing the conversion of MTT to formazan (Sylvester, 2011, Meerloo *et al.*, 2011).

Endothelioma cells were seeded in a 96-well plate at a density of 5 x 10^3 cells/well, and allowed to form a monolayer overnight in a 37°C incubator with 5% CO_2. Endothelioma cells were treated with either LY294,002 at concentrations of 0.01, 0.1, 1 and 10 µM or with PF573,228 at concentrations of 0.01, 0.1, 1 and 10 µg/ml. Controls cells were treated with 0.05% dimethyl sulfoxide (DMSO), a solvent for both drugs. The plate was incubated further for 48 h. After incubation, the media was removed from the wells and the wells were washed once with PBS. Twenty microliters of MTT reagent was added to each well and the plate was incubated for 4 h. Following incubation, the MTT reagent was replaced with 100 µl of an isopropanol-HCl solution and incubated for 1 h. After incubation, the plate was read in a UV plate reader at 570 nm.

2.4. Cell Migration Assay

To study cell migration patterns, experiments were performed using the xCELLigence RTCA DP instrument (Roche Applied Science, Penzberg, Germany). This system has the ability to measure, quantitatively, cell migration in real time (Limame *et al*., 2012). The assay is conducted using a Cell Invasion and Migration (CIM) assay plate that contains a bottom chamber, which houses a chemo-attractant, and an upper chamber where cells are seeded and treated with the drug to be tested. Briefly, three CIM plates were prepared for the study. Medium supplemented with basic Fibroblast Growth Factor (bFGF) was introduced to the bottom chambers of the plates. Twenty five microliters of serum free media was added to the top chamber to hydrate the membranes. The plates were then allowed to equilibrate for 1 h in the

CO_2 incubator at 37°C before taking a background measurement on the RTCA DP instrument. Endothelioma cells were then added to the top chambers of the plates, and the plates were placed back on the RTCA DP instrument in the CO_2 incubator, and cell migration monitored for 24 h.

2.5. Cell Morphology Studies

2.5.1. Light Microscopy

The Hematoxylin and Eosin (H & E) staining method was used to investigate changes in the morphology of endothelioma cells after treatment with LY294,002 for 48 h. This staining method has been used by pathologists to examine tissue samples from cancer patients to aid in cancer diagnosis (Fischer *et al*., 2008). Hematoxylin is a deep blue-purple coloured dye that stains nuclear material blue. Eosin is a pink-orange dye that stains the cytoplasmic material and the extracellular matrix pink (Fischer *et al*., 2008).

Endothelioma cells were seeded on cover slips in 6-well plates at a density of $3x10^5$ cells/ml. After 24 h, the cells were treated with LY294,002 (0 – 10 µM) over a period of 48 h. Control cells were treated with DMSO. After treatment, the cells were fixed in Bouin's fluid for 30 min. This was followed by rinsing the cells in 70% ethanol for a further 30 min, and then rinsed with water. The cells were immersed in Meyer's haemalum for 20 min, rinsed with water for 2 min, followed by 70% ethanol. The cells were incubated for 2 min in 1% eosin and then rinsed twice with increasing concentrations of ethanol (70%, 96%, and 100%). Xylol was used to rinse the cells twice before mounting them onto microscopic glass slides using a resin. The slides were viewed with a light microscope (Olympus, supplied by Wirsam Scientific, South Africa).

2.5.2. Transmission Electron Microscopy

Transmission Electron Microscopy (TEM) is a vital tool for viewing samples at higher resolutions. Since TEM uses electron beams to illuminate the sample instead of light, samples can be analysed at micrometre, nanometre, and even sub-nanometre scales. Transmission Electron Microscopy has been used extensively to investigate apoptosis and other death mechanisms in cells. Previously, it was considered as the gold standard in the investigation of

apoptosis (Elmore, 2007). It remains a useful tool for investigating the morphological features of apoptosis in cells.

Endothelioma cells were seeded in 25 cm^2 flasks at a density of 1.25×10^6 cells per flask and allowed to attach overnight. Cells were treated with LY294,002 and control cells were treated with DMSO. After 48 h, the cells were fixed with 2.5% gluteraldehyde and then centrifuged at 3000 rpm for 3 min. The samples were subjected to 1% osmium tetroxide (OsO4) in Millonig's buffer. After 1 h, samples were incubated in Millonig's buffer for 10 min, and rinsed in distilled water for 20 min. This was followed by rinsing in increasing ethanol concentrations (50%, 70%, 80%, and 96%), and thereafter in 100% ethanol and propylene oxide (PO). The cells were then embedded in a PO:Epoxy resin (2:1), followed by polymerization in 100% epoxy. Samples were viewed using TEM (Apollo Scientific SA, South Africa).

2.6. Caspase Activity Assay

Caspases are a family of cysteine proteases that play essential roles in apoptosis (Wang and Lenardo, 2000). Caspases have been used as biochemical markers for apoptosis since they mediate most biochemical and morphological changes observed in apoptotic cells (Kohler *et al*., 2002). These include chromatin condensation, DNA fragmentation, and membrane blebbing (Kohler *et al*., 2002).

Endothelioma cells were seeded in 25 cm^2 flasks at a density of 1 x 10^6 cells per flask and allowed to attach. Cells were treated with saline or LY294,002. Three wells were analysed per treatment dose. Caspase-3 activity was measured using a caspase-3 ELISA assay kit (BioVision, Mountain View, USA). Cells were incubated in lysis buffer. Afterwards, samples were centrifuged at 16,000 x g for 15 min. The supernatant from each tube was discarded and the pellet was incubated in assay buffer (+ 200 mM substrate) at 37^{o}C for 4 h. The absorbance was read at 405 nm with an ELx 800 Microplate Reader (Bio-Tek instruments Inc, Weltevreden, SA).

2.7. Protein Microarray Studies

Protein microarrays have been successfully applied in protein expression profiling, biomarker identification and clinical diagnosis (Hu *et al*., 2011). They are used for the identification, quantification and functional analyses of several proteins, simultaneously, in a sample (Poetz *et al*., 2005).

Endothelioma cells were seeded on cover slips in 6-well cell culture plates at a density of 3 x 10^5 cells per well. After 24 hours, the cells were treated with LY294,002 (10 µM) over a period of 48 h. The mouse angiogenesis array kit (Ray Biotech Inc., Norcross, USA) was used to study the expression profile of angiogenic proteins in conditioned medium from control and LY294,002-treated cells according to the protocol of the manufacturer. The protein expression was visualized with a FluorChem Imaging System (Proteinsimple, CA, USA).

2.8. Statistical Analysis

The data is presented as mean values ± standard deviation (SD). Data was analysed using one-way analysis of variance (ANOVA), followed by Bonferroni's method (Didelez *et al*., 2006). $P < 0,05$ was considered as statistically significant. Analysis was undertaken using the software package Graph-Pad Prism 6.

The IC_{50} values were determined for both drugs. This is the half-maximum inhibitory concentration, defined as the total concentration of a drug or any other inhibitor required to inhibit a certain biological process by half (Griffiths and Sundaram, 2011). For cell proliferation studies, the IC_{50} values were determined on log transformed data using Graph-Pad Prism 6. For comparison, the IC_{50} values of the drugs are reported in µM. For cell migration studies, the IC_{50} values of the drugs were generated automatically by the xCELLigence software.

Chapter 3

Results

3.1. Cell Viability Assay

The viability of control and drug treated endothelioma cells was studied using the MTT assay. Cell viability was inhibited by both PF573,228 and LY294,002 in a dose dependent manner (Fig. 3.1). It can be observed from this figure that the number of viable cells decreased when the concentration of both drugs was increased. The IC_{50} value was approximately 0.5325 µM for LY294,002 and approximately 0.6589 µM for PF573,228. The IC_{50} measurement for PF573,228 was converted to µM for comparison with that of LY294,002.

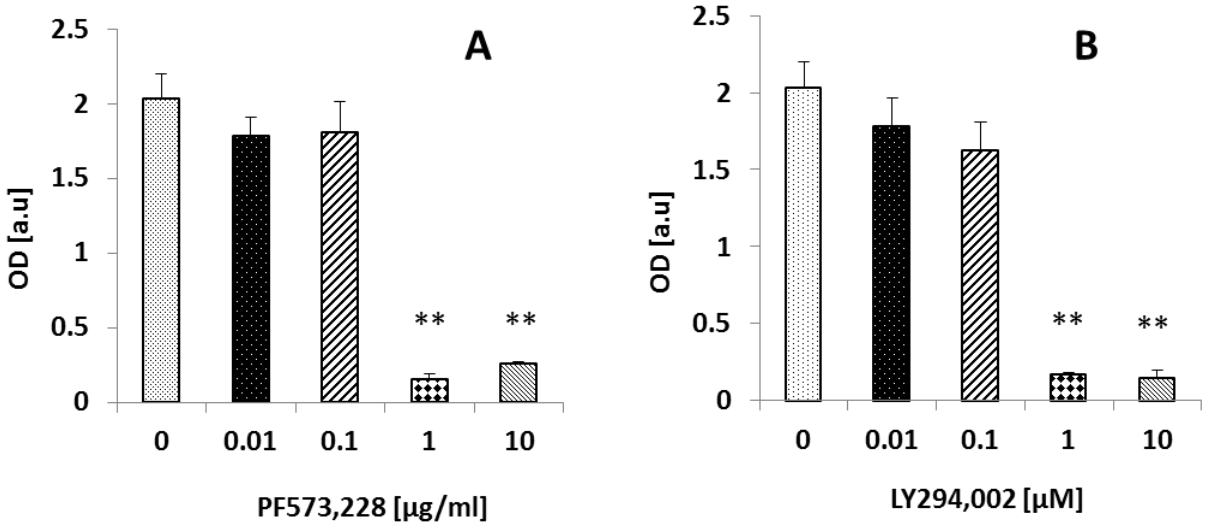

Figure 3.1: Effects of PF573,228 (A) and LY294,002 (B) on the metabolic activity of endothelioma cells. Endothelioma cells were treated with PF573,228 and LY294,002 at concentration ranges of 0.01 µg/ml to 10 µg/ml and 0.01 µM to 10 µM respectively. *P<0.0001 compared to controls (Zero drug concentration). Results are mean OD ±SD.

3.2. Cell Migration Assay

Both PF573,228 and LY294,002 inhibited cell migration in a dose dependent fashion, as shown in Fig. 3.2. A decrease in the amount of cells that have migrated from the top chamber to invade the bottom chamber of the plates was observed as the concentration of the drugs increased. LY294,002, with an IC_{50} value of 3.9425 x 10^{-8} g/ml, seems to have a higher potency compared to PF573,228, which has an IC_{50} value of 6.7143 x 10^{-7} g/ml. This was also observed in Fig. 3.1. Therefore, further studies were undertaken using LY294,002.

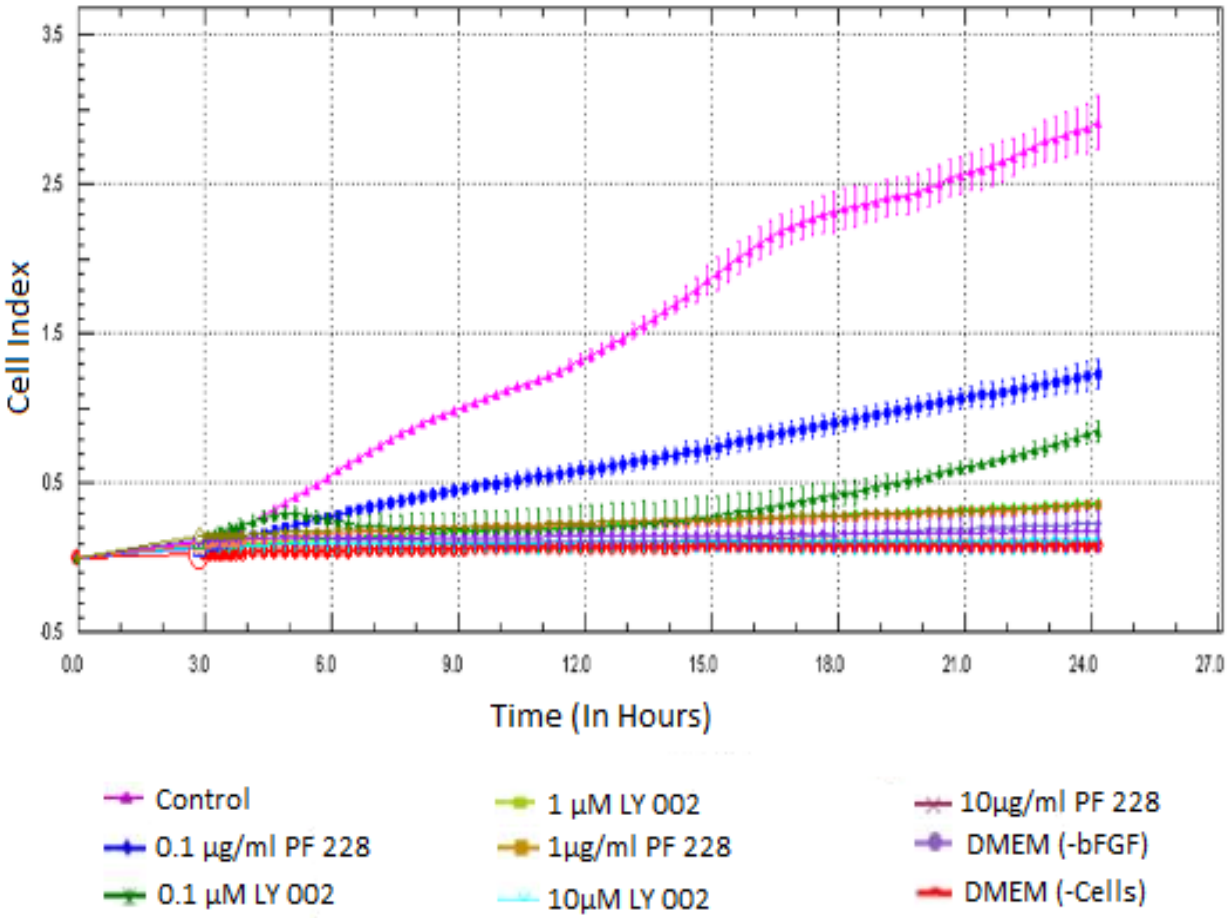

Figure 3.2: Migration of endothelioma cells from control and drug-treated cultures. Endothelioma cells were treated for 24 h with LY294,002 and PF573,228 at a concentration range of 0.01 µg/ml to 10 µg/ml and 0.01 µM to 10 µM respectively. Results are mean CI (Cell Index) ±SD.

Table 3.1: Comparison of IC_{50} values of PF573,228 and LY294,002.

Drug	Cell Activity	IC_{50}
LY294,002	Viability	0.5325 µM
	Migration	1.3663 µM
PF573,228	Viability	0.6589 µM
	Migration	12.83 µM

3.3. Cell Morphology Studies

Based on the observations made in the previous sections, a decision was taken to continue the studies using one drug only, LY294,002, which showed high potency when compared to PF573,228. The morphological changes in endothelioma cells following treatment with LY294,002 were investigated using both light and electron microscopy.

3.3.1. Light Microscopy

Light microscopy results show that control cells had normal morphology (as seen in Fig. 3.3 A & B). Some of the cells were in various stages of mitosis such as metaphase, telophase and cytokinesis. In LY294,002–treated cultures (Fig. 3.3 C & D), there was a decrease in cell density compared to control cells. Also, most cells appeared to have lost cell-cell contact and some have condensed chromatin, which are signs of apoptosis.

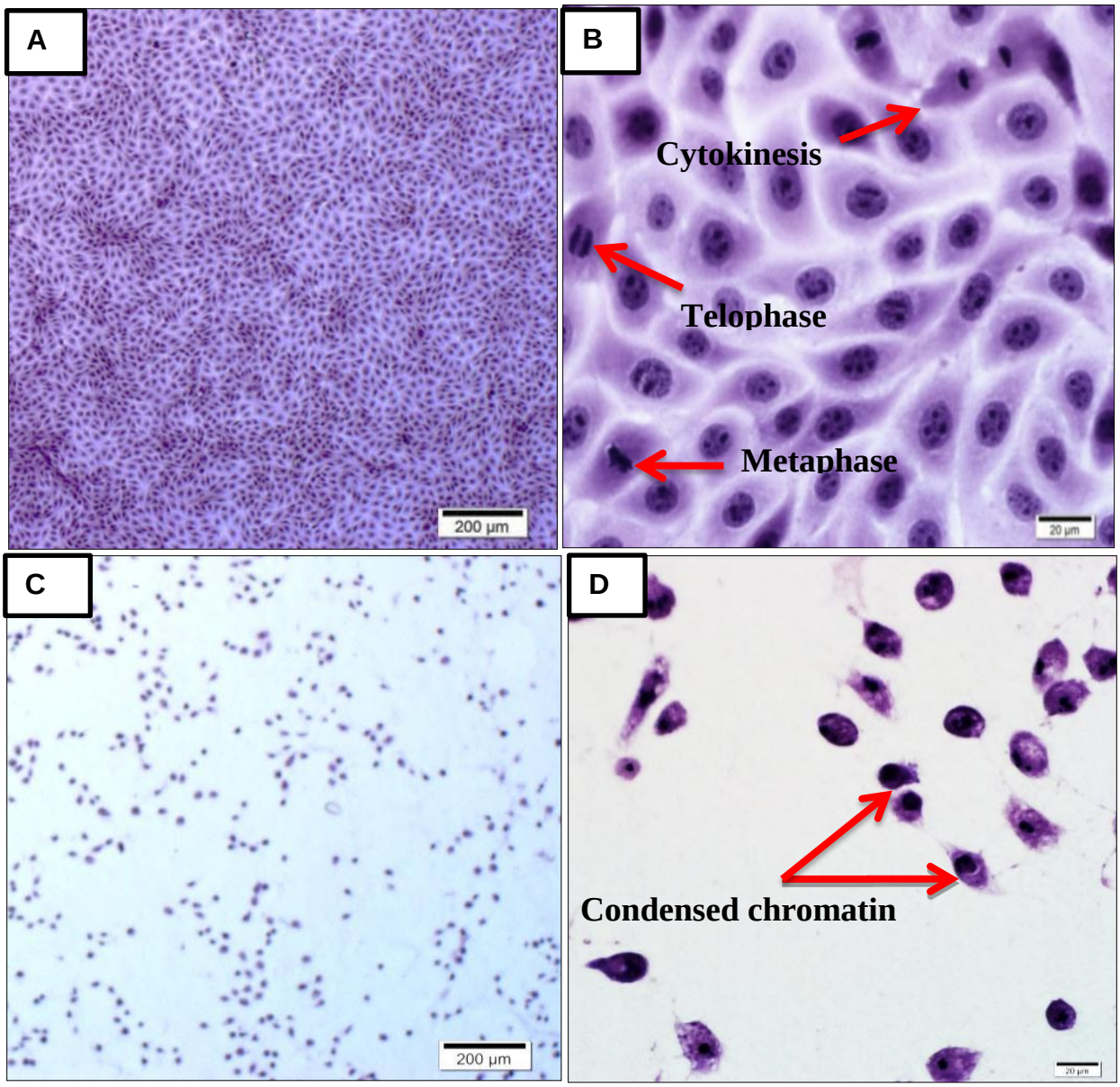

Figure 3.3: H & E staining of endothelioma cells to investigate the effects of LY294,002 on cell morphology. This figure illustrates the differences between non-treated (A & B) and LY294,002 treated (C & D) endothelioma cells. A & C Bar = 200 µm; B & D Bar = 20 µm.

3.3.2. Transmission Electron Microscopy

Further studies on cell morphology were performed using TEM in order to view the interior of individual cells at a much higher magnification, and improved resolution to investigate the differences in the intracellular components of control and LY294,002-treated endothelioma cells that are not easily observed with the light microscope.

Control cultures (Fig. 3.4 A) show a healthy endothelioma cell with typical endothelial cell morphology, while endothelioma cells from drug treated cultures (Fig. 3.4 B) display various changes in morphology. The cells appear round and exhibit chromatin condensation, features that are usually seen in cells undergoing apoptosis. Furthermore, the LY294,002 treated cells appear to have clear zones in the cytoplasm that resemble vacuoles or phagosomes, thereby suggesting possibilities of autophagy. Autophagy is the process in which a cell degrades its unnecessary organelles and proteins as a means to survive stressful situations such as nutrient deprivation (Kung *et al*, 2011).

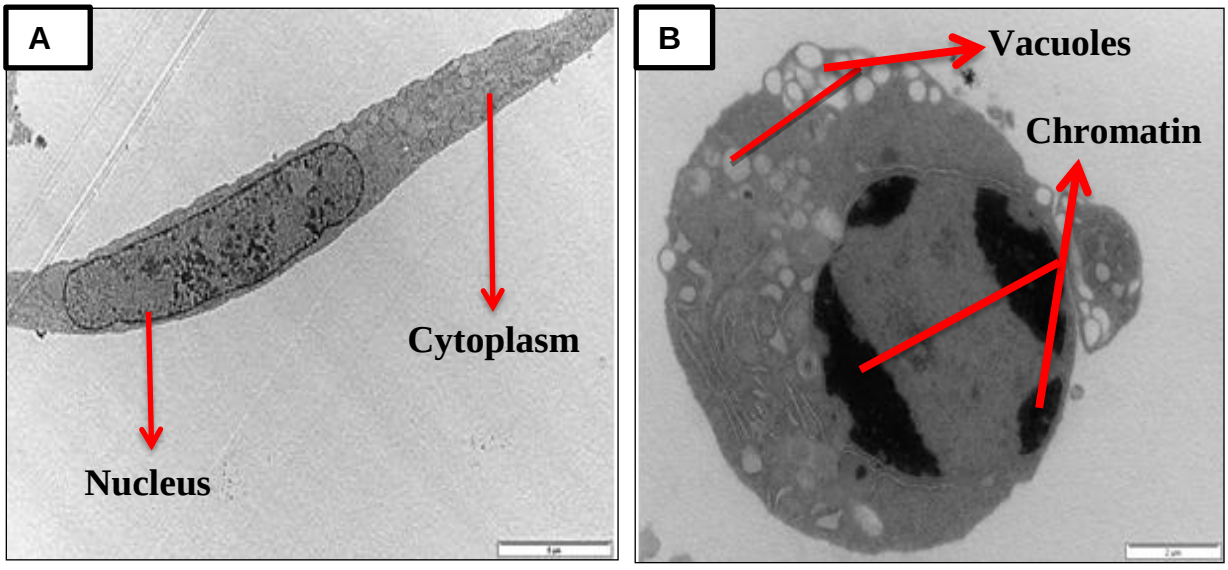

Figure 3.4: Variances in the morphology of intracellular structures of endothelioma cells from control (A) and drug treated (B) endothelioma cell cultures, imaged using transmission electron microscopy. Bar = 2 µm.

3.4. Caspase Activity Assay

To confirm the apoptotic effects of LY294,002 observed in section 3.3, caspase activity was measured. There was an increase in the activation of caspase-3 in endothelioma cell cultures following treatment with LY294,002 compared to the control cultures (Fig. 3.5) ($p<0.001$). Such an increase in caspase-3 activation was not observed in cells treated with a combination of LY294,002 and carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone (Z-VAD-FMK) ($P<0.05$), a potent caspase inhibitor. Since caspases are a family of cysteine proteases which play a role in the execution of apoptosis, these observations therefore indicate that the drug induced apoptosis in endothelioma cells.

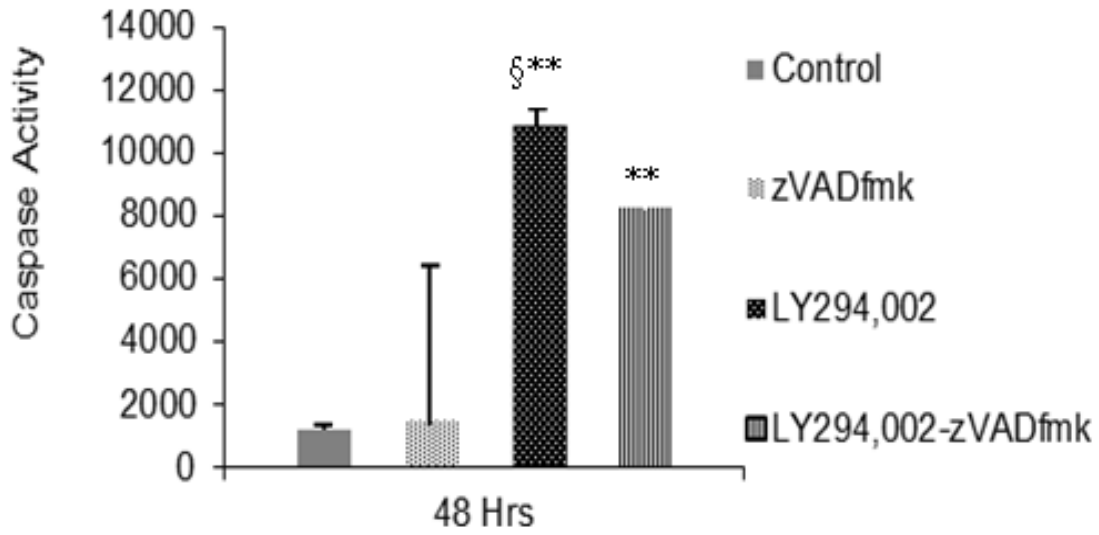

Figure 3.5: LY294,002 increases the activity of caspases-3 in endothelioma cells. Cytosolic lysates were prepared from saline- or LY294,002-treated ECs. Three wells were analysed per treatment dose. Values are mean ± SD from two separate experiments. §$P<0.05$ compared to Z-VAD-FMK; ** $P<0.001$ compared to control.

3.5. Protein Microarray Studies

To determine the anti-angiogenic effects of LY294,002, the expression of angiogenic proteins, both pro- and anti-angiogenic, by endothelioma cells following treatment with the drug was investigated. An increase in the expression of platelet factor 4 (PF-4) and Fas ligand (Fas-L) was observed in conditioned medium from LY 294,002-treated cells. Also, reduced expression of eotaxin-1 and bFGF and complete inhibition of Leptin and VEGF expression was noted in treated endothelioma cell cultures (Fig. 3.6B). A list of the proteins affected by treatment with LY294,002 is shown in Table 3.3. All these proteins are involved in the process of angiogenesis. The effects on these proteins demonstrate that LY294,002 may prevent angiogenesis partly by interfering with the expression of pro- and anti-angiogenic cytokines.

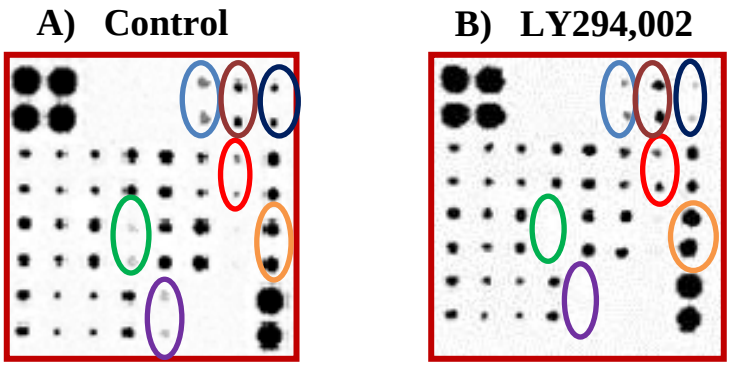

Figure 3.6: The effects of LY294,002 on the angiogenic cytokine profile of endothelioma cells. The names of cytokines are listed in Table 3.2.

Table 3.2: List of angiogenic cytokines for the array studies. POS = Positive Control; NEG = Negative Control; BLANK = Blank Spot

	A	B	C	D	E	F	G	H
1	POS	POS	NEG	NEG	BLANK	Eotaxin-1	Fas Ligand	bFGF
2	POS	POS	NEG	NEG	BLANK	Eotaxin-1	Fas Ligand	bFGF
3	G-CSF	GM-CSF	IFN-gamma	IGF-2	IL-1 alpha	IL-1 beta	IL-12p40/70	IL-12p70
4	G-CSF	GM-CSF	IFN-gamma	IGF-2	IL-1 alpha	IL-1 beta	IL-12p40/70	IL-12p70
5	IL-13	IL-6	IL-9	Leptin	MCP-1	M-CSF	MIG	PF-4
6	IL-13	IL-6	IL-9	Leptin	MCP-1	M-CSF	MIG	PF-4
7	TIMP-1	TIMP-2	TNF-alpha	THPO	VEGF	BLANK	BLANK	POS
8	TIMP-1	TIMP-2	TNF-alpha	THPO	VEGF	BLANK	BLANK	POS

Table 3.3: Changes in the expression of angiogenic cytokines after treatment of endothelioma cell with LY294,002.

Cytokines expressed in control cells	Expression after LY294,002 treatment
IL-12 (p40/70)	Enhanced
Fas L	Enhanced
PF-4	Enhanced
Eotaxin-1	Suppressed
bFGF	Suppressed
Leptin	Blocked
VEGF	Blocked

Chapter 4

Discussion

The metastasis of tumour cells accounts for about 90% of deaths in cancer patients. One of the processes that are central to metastasis, angiogenesis, is receiving attention as a potential target for anti-cancer therapy (Mehlen and Puisieux, 2006). In this study, the effects of small molecule inhibitors (LY294,002 and PF573,228) on the proliferation and migration of endothelial cells derived from vascular tumours (endothelioma cells) were investigated.

Both drugs exhibited anti-proliferative activity in a dose dependent manner, with LY294,002 showing a slightly higher non-proliferative activity compared to PF573,228. The inherent ability of LY294,002 to inhibit cell proliferation was reported in literature by Hu *et al* (2000) when they noticed that LY294,002 inhibited growth of an ovarian carcinoma, *in vivo*, in athymic mice that were inoculated with OVCAR-3 cells. They further specified that, *in vitro*, LY294,002 inhibited proliferation of OVCAR-3 cells. A similar trend was reported by Jiang *et al* (2010) when they showed a dose dependent decrease in the proliferation of a CNE-2Z cell line, using the MTT assay.

The anti-proliferative effects of PF573,228 were previously reported by Cabrita and colleagues (2011). They showed a dose dependant decrease in the proliferation of human umbilical vein endothelial cells (HUVECs) when treated with PF573,228 at a concentration of 0.5 mM, and complete inhibition when the concentration was raised to 10 mM.

According to Hu *et al* (2000), LY294,002 inhibits cell proliferation in either one of two mechanisms. Firstly, LY294,002 exhibits anti-proliferative activity by inducing specific G1 arrest which results in the inhibition of cell cycle progression. This was also confirmed by Gong and colleagues (2012) who reported that LY294,002 inhibits PI3K activity in cancer stem-like cells by blocking cell cycle progression through the G0/G1 arrest. The second mode of inhibition occurs through the activation of apoptosis. LY294,002 promotes apoptosis by inactivating PI3K, thereby disrupting the signalling cascade that helps cells to avoid apoptosis (Hu *et al.*, 2000).

In this study the effects of LY294,002 and PF573,228 on endothelioma cell migration were also investigated. Migration is a very important phenomenon in processes such as embryogenesis, wound healing and inflammation (Chernoivanenko *et al.*, 2012; Valster *et al.*, 2005). However, cell migration occurs during both angiogenesis and metastasis. During metastasis, it facilitates the distribution of cancerous cells from malignant tumours, resulting in the formation of secondary tumours in distant organs (Yamaguchi *et al.*, 2005).

The migration of endothelioma cells was inhibited by both drugs in a dose dependent manner, with LY294,002 being the most potent of the two drugs. LY294,002 inhibited cell migration through its direct effect on PI3K, which is an important modulator of cell migration. This correlates with published data obtained using a different cell line. Sliva and colleagues (2002) reported an LY294,002 assisted inhibition of MDA-MB-231 cell migration by suppressing PI3K activity through binding to the ATP binding site of P110 thereby inducing catalytic inactivation. The regulation of cell migration by PI3K is achieved by two ways; by direct binding of proteins to their lipid products and also by initiating crosstalk with other pathways including Rho GTPase signalling (Cain and Ridley, 2009). It is possible that PF573,228 inhibited migration through inhibiting FAK activity.

Cabrita and colleagues (2011) also tested various FAK inhibitors as anti-angiogenic agents and showed that PF573,228 inhibited HUVEC migration in a dose dependant manner. It has been shown that cells with decreased FAK activity are less capable of migrating even in the presence of a chemotactic or haptotatic stimulus, since FAK primarily regulates migration (Hauck *et al.*, 2002; Parsons, 2003).

The effects of LY294,002 on cell morphology were studied using LM and TEM. Subsequent studies were performed using LY294,002 as this drug had a higher potency when compared to PF573,228. Several changes in morphology were observed between LY294,002-treated endothelioma cells and control cells. Endothelioma cells displayed morphological features that place them at various stages of mitosis such as metaphase, telophase and cytokinesis, suggesting that cells were actively growing and dividing. Contrary, LY294,002-treated cells exhibited signs of apoptosis. These include loss of cell-to-cell contact and chromatin condensation (pyknosis).

These are typical morphological characteristics of apoptotic cells seen using light microscopy (Elmore, 2007).

The observations from both the LM and TEM indicated the ability of LY294,002 to induce cell death through the induction of apoptosis. These findings are in line with earlier work done on LY294,002. Gong *et al* (2012) reported an increased rate of apoptosis in LY294,002 treated cancer stem-like cells where they hypothesised LY294,002 as the driving factor in the induction of apoptosis (Xing *et al.*, 2008). Also, LY294,002 was shown to result in the activation of apoptosis inducing molecules such as p53, and the inactivation of apoptosis suppressing molecules (Xing *et al.*, 2008). It is also possible to assume that the cells might have been in the process of autophagy, however, this would require further studies. According to literature, autophagy is usually activated by cells as a protective measure to block apoptosis. However, excessive autophagy can lead to apoptosis and other programmed cell death processes (Nishida *et al*., 2008; Xing *et al.*, 2008).

The caspase assay was performed to confirm morphological observations, that LY294,002 induced apoptosis in LY294,002-treated endothelioma cells. Caspase-3 is regarded as an effector molecule that initiates the process of apoptosis and thus has been used extensively as a marker to confirm cells undergoing apoptosis (Kohler *et al*., 2002). In this study, an increase in caspase-3 activity was noted in cells treated with LY294,002. Caspase-3 activity from LY294,002-treated cultures was almost five times higher than that observed in control cultures. When a caspase inhibitor (Z-VAD-FMK) was used in combination with LY294,002, a slight decrease in caspase-3 activity was observed, but the activity was still higher than that observed in control cultures. This confirms the observation that LY294,002 induced apoptosis in treated endothelioma cells.

Array studies showed that the expression of pro- and anti-angiogenic cytokines in LY294,002-treated endothelioma cells was altered compared to control endothelioma cells. The three cytokines (platelet factor-4, interleukin-12 and Fas ligand) with an increase in expression following treatment with LY294,002 have been shown to elicit anti-tumour activity. The platelet factor-4 (PF-4) has been reported to induce apoptosis and also inhibit migration and proliferation of endothelial cells by interacting with angiogenesis growth factors (VEGF and FGF) to block

them from binding to their cell surface receptors (Pilatova *et al.*, 2013; Liang *et al.*, 2012; Yamaguchi *et al.*, 2005). Platelet factor-4 was also shown by Liang *et al* (2013) to induce myeloma cell apoptosis and inhibit angiogenesis through the suppression of signal transducer and activation of transcription 3 (STAT3) activity.

Interleukin-12 (IL-12 p40/70), a heterodimer that consists of a light chain (35KDa) and a heavy chain (40KDa), plays an important role in innate and adaptive immunity (Colombo and Trichien, 2002). Interleukin-12 has been shown to have potent anti-tumour activity by stimulating the production of interferon-γ (IFN-γ) by T cells, natural killer cells and natural killer T cells. Interferon-γ then induces the IFN-γ inducible-protein-10 (IP-10) to exert its anti-angiogenic effects in tumour cells (Pan *et al*., 2012).

Fas ligand (Fas-L) is a type II transmembrane protein that acts as a ligand for the apoptosis inducing receptor, Fas (Cataldo *et al.*, 2000). When Fas-L binds to Fas, it elicits a series of signal transduction reactions which result in the activation of apoptosis, termed activation-induced cell death (AICD) (Maher *et al*., 2002). Fas-L was shown by Liu *et al* (2009) to directly induce cytotoxicity against tumour cells, when they successfully tested the use of Fas-L as an anticancer agent in hepatocellular carcinoma. Kim *et al* (2006) also showed that Fas-L induces apoptosis in endothelial cells and leukocytes.

The decrease in the expression of eotaxin-1 and bFGF, and the blockade of VEGF and Leptin expression were also observed following treatment with LY294,002. Eotaxin-1 is a cytokine of the CC chemokine family, which has been reported to promote cancer progression. Levina and colleagues (2009) showed that eotaxin-1 induced migration and invasion of ovarian carcinoma (OVCAR-3) cells. They also showed that eotaxin-1 is capable of stimulating the expression of other cytokines (such as IL-6R and -8), growth factors (such as VEGF) and adhesion molecules (ICAM-1). Eotaxin-1 therefore elicits its proliferative effects either directly or by inducing the production of cytokines, growth factors and adhesion molecules. Eotaxin-1 was also reported by Miyagaki and co-workers (2011) to induce proliferation of EL-4 cells. They also noted that eotaxin-1 enhances cell survival in Ki-JK cells by promoting the expression of anti-apoptotic proteins such as Bcl-xl. According to literature, LY294,002 has little effect on eotaxin-1, since LY294,002 carries out its anti-angiogenic effects through the inhibition of PI3K, and eotaxin-1

induced processes are not mediated by PI3K, hence eotaxin-1 expression was not highly inhibited like that of VEGF, Leptin or bFGF.

When pro-angiogenic growth factors, VEGF and bFGF, are secreted, they stimulate the production of proteases and plasminogen activators which lead to the degradation of the vessel BM causing cells to migrate into the surrounding matrix (Cross and Cleasson-Welsh, 2001). Uncontrolled secretion of VEGF and FGF induce uncontrolled endothelial cell proliferation observed in tumour neovascularisation and in angiogenesis dependant diseases (Presta *et al.*, 2005). LY294,002 has been shown to suppress the expression of VEGF and FGF through the reduction of PI3K activity (Hu *et al.*, 2005). Skinner *et al* (2004) showed that the inhibition of PI3K activity resulted in decreased VEGF transcriptional activation in human OVCAR-3 cells, and also showed that in the presence of forced Akt expression, the inhibitory effect is completely reversed.

Leptin is an adipose derived cytokine that regulates body weight and energy balance in the hypothalamus. Leptin is also produced in solid tumours, under hypoxic conditions, where it elicits pro-angiogenic effects. It has also been shown to enhance endothelial cell growth and suppress apoptosis by increasing the expression of anti-apoptotic proteins such as Bcl-2 and XIAP (Garofalo and Surmacz, 2006). Leptin acts through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway which then induce PI3K to stimulate cellular growth, migration and invasion (Dutta *et al*., 2012).

The activity of LY294,002 and PF573,228 as anti-cancer agents is comparable to that of other molecules known to exhibit anti-cancer properties. These include molecules such as the anti-LRP/LR specific antibodies, W3 and IgG1-iS18, which have been shown to inhibit angiogenesis in-vitro by blocking laminin-1 from binding to its receptor, LRP/RP (Chetty *et al*., 2014, Khusal *et al*., 2013). Furthermore, these antibodies are capable of halting metastatic spread of tumours as they are capable of disrupting the interaction between laminin-1 and LRP/LR which augments the tumorigenic and metastatic potential of tumour cells (Omar et al., 2012). Similar to LY294,002 and PF573,228, anti-LRP/LR antibodies have the potential to be used as tools for therapeutic targeting in cancer therapy.

Chapter 5

Conclusion

One of the major objectives in cancer research is the identification of innovative and effective biomarkers that mediate tumour angiogenesis. Such biomarkers will assist in the early detection of the disease and also in treatment through targeted drug delivery which reduces side effects and further improves efficacy of treatment. In this study, the expression of biomarkers in tumour endothelial cells, and the expression of such biomarkers in response to treatment with anti-cancer compounds were investigated. Two compounds were tested, namely the PI3K inhibitor, LY294,002, and the FAK inhibitor, PF573,228. Tests were performed using vascular endothelial cells derived from tumours of mice. Initially, the effect of the drugs on cell proliferation and migration were tested. This was conducted to investigate the anti-angiogenic properties of the drugs, while also determining their cytotoxicity levels on endothelioma cells.

The treatment of endothelial cells derived from mouse vascular tumours with the drugs disrupted cell proliferation and cell migration. LY294,002, the most potent of the two drugs, further induced apoptotic effects. Increased endothelial cell growth and migration are required during the angiogenesis process and for metastatic spread of tumour cells to a secondary site. Therefore, LY294,002 exhibited anti-angiogenic properties and apoptosis.

Identification of angiogenic factors that are suppressed by LY294,002 to elicit its anti-angiogenic effects could be used as targets in cancer therapy. In comparison to control cultures, numerous changes in the expression of various cytokines were observed. These angiogenic factors are all involved in the process of angiogenesis, and this further confirms their importance in the process. The modification of the expression levels of various cytokines by LY294,002 signifies its anti-angiogenic and anti-tumour capabilities. LY294,002 orchestrated an increase in the expression of cytokines known to drive anti-angiogenic processes, and also suppressed the expression of cytokines known to enhance angiogenesis and metastasis.

Chapter 6

Future Studies

Future studies should involve further investigation of the biomarkers identified in this study as diagnostic markers, or therapeutic targets in cancer. Morphology studies revealed characteristics of autophagy, further studies (such as exploring the expression of the *LC3* gene) should be conducted to investigate the effects of LY294,002 on the activation of other cell death mechanisms. Autophagy in particular promotes drug resistance in cancer, and therefore can be a significant target for anticancer therapy. LY294,002 showed great anti-proliferative and anti-angiogenic activity, however, due to recent reports on the toxicity encountered with the drug in clinical trials, future studies should focus on exploring the conjugation of this drug with nanoparticles to reduce toxicity and enhance therapeutic effect.

Chapter 7

References

Baillie, C. T. Winslet, M. C and N. J Bradley. Tumour vasculature a potential therapeutic target. *British Journal of Cancer* **72** (1995) 257 – 267.

Belson, M. Kingsley, B and A. Holmes. Risk factors for acute leukemia in children: a review. *Environmental Health Perspectives* **115** (2007) 138 – 45.

Berrie, P. C. Phosphoinositide 3-kinase inhibition in cancer treatment. *Expert Opinion* on *Investigational Drugs* **10** (2001) 1085 – 1098.

Bhat, T. A. and R. P. Singh. Tumour angiogenesis – a potential target in cancer chemoprevention. *Food and Chemical Toxicology* 46 (2008) 1334 – 1345.

Bouis, D. Kusumanto, Y. Meijer, C. Mulder, N. H. and G. A. P. Hospers. A review on pro- and anti-angiogenic factors as targets of clinical intervention. *Pharmacological Research* **53** (2006) 89 – 103.

Boya, P. Gonza´lez-Polo, R-A. Casares, N. Perfettini, J - L. Dessen, P. Larochette, N. Me´tivier, D. Meley, D. Souquere, S. Yoshimori, T. Pierron, G. Codogno, P and G. Kroemer. Inhibition of macro-autophagy triggers apoptosis. *Molecular and Cell Biology* **3** (2005) 1025 – 1040.

Burrell, K and G. Zadeh. Molecular mechanisms of tumour angiogenesis. Tumour Angiogenesis. Dr. Sophia Ran (Ed.), ISBN: 978-953-51-0009-6, InTech. Available from: http://www.intechopen.com/books/tumour-angiogenesis/molecular-mechanisms-of-tumour-angiogenesis.

Cabrita, M. A. Jones, L. M. Quizia, J. L. Sabourina, L. A. McKaya, B. C and C. L. Addisona. Focal adhesion kinase inhibitors are potent anti-angiogenic agents. *Molecular Oncology* **5** (2011) 517 – 526.

Cain R. J and A. J. Ridley. Phosphoinositide 3-kinases in cell migration. *Biology of the Cell* **101** (2009) 13–29.

Cataldo, N. A. Dumesic, D. A. Goldsmith, P. C and R. B Jaffe. Immunolocalisation of Fas and Fas ligand in the ovaries of women with polycystic ovary syndrome: relationship to apoptosis. *Human Reproduction* **15** (2000) 1889-1897.

Cavallaro, U and G. Christofori. Molecular mechanisms of tumour angiogenesis and tumour progression. *Journal of Neuro-Oncology* **50** (2000) 63 – 70.

Chavakis, E and S. Dimmeler. Regulation of endothelial cell survival and apoptosis during angiogenesis. *Arteriosclerosis, Thrombosis,* and *Vascular Biology* **22** (2002) 887 – 893.

Chen, M. H. Kerkelä, R and T. Force. Mechanisms of Cardiac Dysfunction Associated With Tyrosine Kinase Inhibitor Cancer Therapeutics. *Circulation.* **118** (2008) 84 – 95.

Chernoivanenko, I. S. Minin, A. A and A. A. Minin. Role of vimentin in cell migration. *Russian Journal of Developmental Biology* **44** (2013) 144–157.

Chetty, C. Khumalo, T. Da Costa Dias, B. Reusch, U. Knackmuss, S. Little, M and S. F. T. Weiss. Anti-LRP/LR specific antibody IgG1-iS18 impedes adhesion and invasion of liver cancer cells. *Public Library of Science One* **9** (2014) 1 – 10.

Cooper, G. M. The Cell: A Molecular Approach. 2nd edition. Sunderland (MA): Sinauer Associates; 2000. The Development and Causes of Cancer. Available from: http://www.ncbi.nlm.nih.gov/books/NBK9963

Courtney, K. D. Corcoran, R. B. and J. A. Engelman. The PI3K pathway as a drug target in human cancer. *Journal of Clinical Oncology* **28** (2010) 1075 – 1083.

Cross, M. J. and L. Cleasson-Welsh. FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. *Trends in Pharmacological Sciences* **22** (2001) 201 – 207.

Didelez, V. Pigeot, I and P. Walter. Modifications of the Bonferroni-Holm procedure for a multi-way ANOVA. *Statistical Papers* **47** (2006) 181 – 209.

DuBois, S. and G. Demetri. Markers of angiogenesis and clinical features in patients with sarcoma. *American Cancer Society* **109** (2007) 813 – 819.

Dudley, A. C. Tumour endothelial cells. *Cold Spring Harbor Perspectives in Medicine* **2** (2012) 1 – 28.

Dunlop, R. J. and C. W. Campbell. Cytokines and advanced cancer. *Journal of Pain and Symptom Management* **20** (2000) 214 – 232.

Dutta, D. Ghosh, S. Pandit, K. Mukhopadhyay, P and S. Chowdhury. Leptin and cancer: Pathogenesis and modulation. *Indian Journal of Endocrinology and Metabolism* **16** (2012) 596-600

Edinger, A. L and C. B. Thompson. Death by design: apoptosis, necrosis and autophagy. *Current Opinion in Cell Biology*. **16** (2004) 663-669.

Eichhorn, M. E. Kleespies, A. Angele, M. K. Jauch, K.W. and C. J. Bruns. Angiogenesis in cancer: molecular mechanisms, clinical impact. *Langenbeck's Archives of* Surgery **392** (2007) 371 – 379.

Elmore, S. Apoptosis: a review of programmed cell death. *Toxicologic Pathology* **35** (2007) 495 – 516.

Farber, E. The multistep nature of cancer development. *Cancer Research* **44** (1984) 4217 – 4223.

Ferlay, J. Shin, H-R. Bray, F. Forman, D. Mathers, C and D.M. Parkin. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *International Journal of Cancer* **127** (2008) 2893 – 2917.

Ferlay, J. Soerjomataram, I, Ervik, M. Dikshit, R. Eser, S. Mathers, C. Rebelo, M. Parkin, D. M. Forman, D and F. Bray. Cancer Incidence and Mortality Worldwide: GLOBOCAN 2012. *International Agency for Research on Cancer* **11** (2013) 1133-1145

Fidler, I. J. Critical determinants of metastasis. *Seminars in Cancer Biology* **12** (2002) 89 – 96.

Fischer, A. H. Jacobson, K. A. Rose, J and R. Zeller. Hematoxylin and eosin staining of tissue and cell sections. *Cold Spring Harbor Protocols* **10** (2008) 63 – 78.

Folkman, J. Angiogenesis and apoptosis. *Seminars in Cancer Biology* **13** (2003) 159 – 167.

Francavilla, C. Maddaluno, L. and U. Cavallaro. The functional role of cell adhesion molecules in tumour angiogenesis. *Seminars in Cancer Biology* **19** (2009) 298 – 309.

Frankenberry, K. A. Somasundar, P. McFadden, D. W and L. C. Vona-Davis. Leptin induces cell migration and the expression of growth factors in human prostate cancer cells. *The American Journal of Surgery* **188** (2004) 560 – 565.

Fu, W. Hall, J. E and M. D. Schaller. Focal adhesion kinase-regulated signaling events in human cancer. *Biomolecular Concepts* **3** (2012) 225 – 240.

Gan, H. K and L. L. Siu. Focal adhesion kinase as a therapeutic target in cancer. *American Society of Clinical Oncology* **45** (2009) 130 – 137.

Garofalo, C and E. Surmacz. Leptin and cancer. *Journal Of Cellular Physiology* **207** (2006) 12 – 22.

Geiger, T. R. and D. S. Peeper. Metastasis mechanisms. *Biochimica et Biophysica Acta* **1796** (2009) 293 – 308.

Gerber, D. E. Targeted therapies: a new generation of cancer treatments. *American Family Physician* **77** (2008) 312 – 319.

Givant-Horwitz, V. Davidson, B and R. Reich. Laminin-induced signaling in tumor cells. *Cancer Letters* **223** (2005) 1 – 10.

Gong, C. Liao, H. Wang, J. Lin, Y. Qi, J. Qin, L. Tian, L-Q and F-J. Guo. LY294002 induces G0/G1 cell cycle arrest and apoptosis of cancer stem-like cells from human osteosarcoma via down-regulation of PI3K activity. *Asian Pacific Journal of Cancer Prevention* **13** (2012) 3103 – 3107.

Gong, J, Morishita, A. Kurokohchi, K. Tani, J. Kato, K. Miyoshi, H. Inoue, H. Kobayashi, M. Liu, S. Murota, M. Muramatsu, A. Izuishi, K. Suzuki, Y. Yoshida, H. Uchida, N. Deguchi, K. Iwama, H. Ishimaru, I and T. Masaki. Use of protein array to investigate receptor tyrosine kinases activated in gastric cancer. *International Journal of Oncology* **36** (2010) 101 – 106.

Gonzalez, R. R. Watters, A. Xu, Y. Singh, U. P. Mann, D. R. Rueda, B. R and M. L. Penichet. Leptin-signalling inhibition results in efficient anti-tumour activity in estrogen receptor positive or negative breast cancer. *Breast Cancer Research* **11** (2009) 1 – 20.

Gonzalez-Perez, R. R. Lanier, V and G. Newman. Leptin's pro-angiogenic signature in breast cancer. *Cancers* **5** (2013) 1140 – 1162.

Griffiths, M and H. Sundaram. Drug design and testing: profiling of anti-proliferative agents for cancer therapy using a cell-based methyl-[3H]-thymidine incorporation assay. *Methods in Molecular Biology* **731** (2011) 451 – 465.

Gupta, M. K and R-Y. Qin. Mechanism and its regulation of tumour-induced angiogenesis. *World Journal of Gastroenterology* **9** (2003) 1144 – 1155.

Haab, B. B. Antibody arrays in cancer research. *Molecular & Cellular Proteomics* **4.4** (2005) 377 – 383.

Hanahan, D and R. A Weinberg. The hallmarks of cancer. *Cell* **100** (2000) 57 – 70.

Hanahan, D and R. A. Weinberg. Hallmarks of cancer: the next generation. *Cell* **144** (2011) 646 – 674.

Hao, H. Naomoto, Y. Bao, X. Watanabe, N. Sakurama, Noma, K. Motoki, T. Tomono, Y. Fukazawa, T. Shirakawa, Y. Yamatsuji, T. Matsuoka, J. Wang, Z. G and M. Takaoka.

Focal adhesion kinase as potential target for cancer therapy (Review). *Oncology Reports* **22** (2009) 973 – 979.

Harlozinska, A. Progress in molecular mechanisms of tumour metastasis and angiogenesis. *Anticancer Research* **25** (2005) 3327 – 3334.

Hashizume, H. Baluk, P. Morikawa, S. McLean, J. W. Thurston, G. Roberge, S. Jain, R. K. and D. M. McDonald. Openings between defective endothelial cells explain tumour vessel leakiness. *American Journal of* Pathology **156** (2000) 1363 – 1380.

Hauck, C. R. Hsia, D. A and D. D. Schlaepfer. The focal adhesion kinase - a regulator of cell migration and invasion. *International Union of Biochemistry and Molecular Biology Life* **53** (2002) 115 – 119.

Hemmings, B. A. and D. F. Restuccia. PI3K-PKB/Akt pathway. *Cold Spring Harbor Perspectives in Biology* **10 (**2012) 1 – 3.

Hu, L. Hofmann, J and R. B. Jaffe. Phosphatidylinositol 3-Kinase mediates angiogenesis and vascular permeability associated with ovarian carcinoma. *Clinical Cancer Research* **11** (2005) 8208 – 8212.

Hu, L. Zaloudek, C. and G. B. Mills. *In-vivo* and *in-vitro* ovarian carcinoma growth inhibition by a phosphatidylinositol 3-kinase inhibitor (LY294,002). *Clinical Cancer Research* **6** (2000) 880 – 886.

Hu, S. Xie, Z. Qian, J. Blackshaw, S and H Zhu. Functional protein microarray technology. *Wiley Interdisciplinary Reviews Systems Biology* and *Medicine* **3** (2011) 255 – 268.

Jemal, A. Bray, F. Center, M. M. Ferlay, J. Ward, E and D. Forman. Global cancer statistics. *CA: A Cancer Journal for Clinicians* **61** (2011) 69 – 90.

Jemal, A. Center, M. M. De Santis, C and E. M. Ward. Global patterns of cancer incidence and mortality rates and trends. *Cancer Epidemiology, Biomarkers & Prevention* **19** (2010) 1893 – 1907.

Jiang, H. Fan, D. Zhuo, G. Li, X. and H. Deng. Phosphatidylinositol 3-Kinase inhibitor (LY294,002) induces apoptosis of human nasopharyngeal carcinoma in vitro and in vivo. *Journal of Experimental & Clinical Cancer Research* **29** (2010) 29 – 34.

Johnson, K. E and T. A. Wilgus. Multiple roles for VEGF in non-melanoma skin cancer: angiogenesis and beyond. *Journal of Skin Cancer* **2012** (2012) 1 – 6.

Khumalo, T. Reusch, U. Knackmuss, S. Little, M. Veale, R. B and S. F. T. Weiss. Adhesion and invasion of breast and oesophageal cancer cells are impeded by anti-LRP/LR-specific antibody IgG1-iS18. *Public Library of Science One* **8** (2013) 1 – 8.

Kim, D. Kim, S. Koh, H. Yoon, S. Chung, A. Cho, K. S and J. Chung. Akt/PKB promotes cancer cell invasion via increased motility and metalloproteinase production. *The Faseb Journal* **15** (2001) 1953 – 1962.

Kim, W-U. Kwok, S-K. Hong, K-H. Yoo, S-A. Kong, J-S. Choe, J and C-l Cho. Soluble Fas ligand inhibits angiogenesis in rheumatoid arthritis. *Arthritis Research & Therapy* **9** (2007) 1 – 9.

Kohler, C. Orrenius, S and B. Zhivotovsky. Evaluation of caspase activity in apoptotic cells. *Journal of Immunological Methods* **265** (2002) 97 – 110.

Lechertier, T and K. Hodivala-Dilke. Focal adhesion kinase and tumour angiogenesis. *The Journal of Pathology* **226** (2012) 404 – 412.

Lee, C. M. Fuhrman, C. B. Planelles, V. Peltier, M. R. Gaffney, D. K. Soisson, A. P. Dodson, M. K. Tolley, H. D. Green, C. L and K. A. Zempolich. Phosphatidylinositol 3-Kinase inhibition by LY294002 radiosensitizes human cervical cancer cell lines. *Clinical Cancer Research* **12** (2006) 250 – 256.

Levina, V. Nolen, B. M. Marrangoni, A. M. Cheng, P. Marks, J. R. Szczepanski, M. J. Szajnik, M. E. Gorelik, E and A. E. Lokshin. Role of eotaxin-1signaling in ovarian cancer. *Clinical Cancer Research* **15** (2009) 1- 22.

Liang, P. Cheng, H. Cheng, C. K. Lau, K. M. Lin, S. Y. Chow, E. Y. D. Chan, N. P. H. Ip, R. K. L. Wong, R. S. M and M. H. L. Ng. Platelet factor 4 induces cell apoptosis by inhibition

of STAT3 via up-regulation of SOCS3 expression in multiple myeloma. *Haematologica* **98** (2013) 288–295

Liang, P. Cheng, S. H. Cheng, C. K. Lau, K. M. Lin, S. Y. Chow, E. Y. D. Chan, N. P. H. Ip, R. K. L. Wong, R. S .M and M. H. L. Ngi. Platelet factor 4 induces cell apoptosis by inhibition of STAT3 via up-regulation of SOCS3 expression in multiple myeloma. *Haematological* **98** (2013) 288 – 295.

Liao, D and R. S. Johnson. Hypoxia: a key regulator of angiogenesis in cancer. *Cancer and Metastasis Reviews* **26** (2007) 281 – 290.

Liekens, S. De Clercq, E. and J. Neyts. Angiogenesis: regulators and clinical applications. *Biochemical Pharmacology* **61** (2001) 253 – 270.

Limame, R. Wouters, A. Pauwels, B. Fransen, E. Peeters, M.Lardon, F. De wever, O and P. Pauwels. Comparative analysis of dynamic cell viability, migration and invasion assessments by novel real-time technology and classic endpoint assays. *PLoS ONE* **7** (2012) 1 – 12.

Liu, Z. Wang, J. Yin, P. Qiu, J. Liu, R. Li, W. Fan, X. Cheng, X. Chen, C. Zhang, J and G. Zhuang. RGD-FasL induces apoptosis in hepatocellular carcinoma. *Cellular & Molecular Immunology* **6** (2009) 285 – 293.

Lou, Z. Ren, T. Peng, X. Sun, Y. Jiao, G. Lu, Q. Zhang, S. Lu, X and W. Guo. Bortezomib induces apoptosis and autophagy in osteosarcoma cells through in vitro mitogen-activated protein kinase pathway. *Journal of International Medical Research* **41** (2013) 1505 – 1519.

Luo, J. Manning, B. D. and L. C. Cantley. Targeting the PI3K-Akt pathway in human cancer: Rationale and promise. *Cancer Cell* **4** (2003) 257 – 262.

Mabeta, P and M. S. Pepper. A comperative study on the anti-angiogenic effects of DNA-damaging and cytoskeletal-disrupting agents. *Angiogenesis* **12** (2009) 81 – 90.

Maher, S. Toomey, D. Condron, C and D. Bouchier-Hayes. Activation-induced cell death: the controversial role of Fas and Fas ligand in immune privilege and tumour counterattack. *Immunology and Cell Biology* **80** (2002) 131 – 137.

Majewski, S. Marczak, M. Szmurlo, A. Jablonska, S and W Bollag. Interleukin-12 inhibits angiogenesis induced by humor tumour cell lines *in-vivo*. *The Society for Investigative Dermatology* **106** (1996) 1114 – 1118.

Matsumoto, T. Miyazaki, S. Matsuyama, S. Takeda, M. Kawano, M. Nakagawa, H. Nishimura, K and S. Matsuo. Selection of autophagy or apoptosis in cells exposed to ER-stress depends on ATF4 expression patter n with or without CHOP expression. *Biology Open* **10** (2013) 1 – 7.

Mavria, G. Vercoulen, Y. Yeo, M. Paterson, H. Karasarides, M. Marais, R. Bird, D. and C. J. Marshall. ERK-MAPK signalling opposes Rho-kinase to promote endothelial cell survival and sprouting during angiogenesis. *Cancer Cell* **9** (2006) 33 – 44.

McNamara, C. R. and A. Degterev. Small-molecule inhibitors of the PI3K signalling network. *Future Medicinal Chemistry* **3** (2011) 549 - 565.

Mehlen, P and A. Puisieux. Metastasis: a question of life or death. *Nature Reviews Cancer* **6** (2006) 449 - 458.

Menakuru, S. R. Brown, N. J. Staton, C. A and M. W. R. Reed. Angiogenesis in pre-malignant conditions. *British Journal of Cancer* **99** (2008) 1961 – 1966.

Mishra, R. K. Scaife, J. E. Harb, Z. Gray, B. C. Djukanovic, R and G. Dent. Differential dependence of eosinophil chemotactic responses on phosphoinositide 3-kinase (PI3K). *Allergy* **60** (2005) 1204 – 1207.

Miyagaki, T. Sugaya, M. Murakami, T. Asano, Y. Tada, Y. Kadono, T. Okochi, H. Tamaki, K and S. Sato. CCL11–CCR3 interactions promote survival of anaplastic large cell lymphoma cells via ERK1/2 activation. *Cancer Research* **71** (2011) 2056 – 2065.

Moodley, K and S. F. T. Weiss. Down-regulation of the non-integrin laminin receptor reduces cellular viability by inducing apoptosis in lung and cervical cancer cells. *Public Library of Science One* **8** (2013) 1 – 7.

Moses, M. A. The regulation of neovascularization by matrix metalloproteinases and their inhibitors. *Stem Cells* **15 (**1997) 180 – 189.

Mousa, S. A. Cell adhesion molecules: potential therapeutic & diagnostic implications. *Molecular Biotechnology* **38** (2008) 33 – 40.

Neal, C. P. and D. P. Berry. Basic principles of the molecular biology of cancer II: angiogenesis, invasion and metastasis. *Surgery* **24** (2006) 120 – 125.

Neufeld, G. and O. Kessler. Pro-angiogenic cytokines and their role in tumour angiogenesis. *Cancer and Metastasis Reviews* **25** (2006) 373 – 385.

Nishida, K. Yamaguchi, O and K. Otsu. Crosstalk between autophagy and apoptosis in heart disease. *Circulation Research* **10** (2008) 343 – 351.

Nussenbaum, F and I. M. Herman. Tumour angiogenesis: insights and innovations. *Journal of Oncology* **10** (2010) 1 – 24.

Oliveira, P. A. Colaço, A. Chaves, R. Guedes-Pinto, H. De-la-Cruz P, L. F and C. Lopes. Chemical carcinogenesis. *Anais da Academia Brasileira de Ciências* **79** (2007) 593 – 616.

Omar, A. Reusch, U. Knackmuss, S. Little, M and S. F. T. Weiss. Anti-LRP/LR specific antibody IgG1-iS18 significantly reduces adhesion and invasion of metastatic lung, cervix, colon and prostate cells. *Journal of Molecular Biology* **419** (2012) 102 – 109.

Osaki, M. Oshimura, M and H. Ito. PI3K-Akt pathway: Its functions and alterations in human cancer. *Apoptosis* **9** (2004) 667 – 676.

Otrock, Z. K. Hatoum, H. A. Awada, A. H. Ishak, R. S. and A. I. Shamseddine. Hypoxia-inducible factor in cancer angiogenesis: structure, regulation and clinical perspectives. Critical *Reviews in Oncology/Hematology* **70** (2009) 93 – 102.

Pandya, N. M. Dhalla, N. S and D. D. Santani. Angiogenesis - a new target for future therapy. *Vascular Pharmacology* **44** (2006) 265 – 274.

Papetti, M and I. M. Herman. Mechanisms of normal and tumour-derived angiogenesis. *American Journal of Physiology* **282** (2002) 947 – 970.

Parsons, J. T. Focal adhesion kinase: the first ten years. *Journal of Cell Science* **116** (2003) 1409 – 1416.

Parsons, J. T. Slack-Davis, J. Tilghman, R and W. G. Roberts. Focal adhesion kinase: targeting adhesion signalling pathways for therapeutic intervention. *Clinical Cancer Research* **14** (2008) 627 – 632.

Pepper, M. S. Role of matrix metalloproteinase and plasmogen activator-plasmin systems in angiogenesis. *Journal of the American Heart Association* **21** (2001) 1104 – 1117.

Pilatova, K. Greplova, K. Demlova, R. Bencsikova, B. Klement, G. L and L. Zdrazilova-Dubska. Role of platelet chemokines, PF-4 and CTAP-III, in cancer biology. *Journal of Hematology & Oncology* **6** (2013) 1 – 8.

Pitot, H. C and Y. P. Dragan. Facts and theories concerning the mechanisms of carcinogenesis. *The FASEB Journal* **5** (1991) 2280 – 2286.

Plank, M. J. and B. D. Sleeman. Tumour-induced angiogenesis: a review. *Journal of Theoretical Medicine* **5** (2003) 137 – 153.

Poetz, O. Schwenk, J. M. Kramer, S. Stoll, D. Templin, M. F and T. O. Joos. Protein microarrays: catching the proteome. *Mechanisms of Ageing and Development* **126** (2005) 161–170.

Popat, K. McQueen, K and T. W. Feeley. The global burden of cancer. *Best Practice & Research Clinical Anaesthesiology* **27** (2013) 399 – 408.

Porter, A. G and R. U Janicke. Emerging roles of caspase-3 in apoptosis. *Cell Death and Differentiation* **6** (1999) 99-104.

Potente, M. Urbich, C. Sasaki, K. Hofmann, W. F. Heeschen, C. Aicher, A. Kollipara, R. DePinho R. A. Zeiher, A. M, and S. Dimmeler. Involvement of FOXO transcription factors in

angiogenesis and postnatal neovascularization. *The Journal of Clinical Investigation* **115** (2005) 2382 – 2392.

Presta, M. Dell 'Era, P. Mitola, S. Moroni, E. Ronca, R. and M. Rusnati. Fibroblast Growth Factor/Fibroblast Growth Factor receptor system in angiogenesis. *Cytokine & Growth Factor Reviews* **16** (2005) 159 – 178.

Pugh, C. W. and P. J. Ratcliffe, Regulation of angiogenesis by hypoxia role of the HIF system. *Nature Medicine* **9** (2003) 677 – 684.

Radic, S. Stanojevic, Z and B. Dindic. The pathogenesis of neoplasia. *Archive of Oncology* **12** (2004) 35 – 37.

Ramjaun, A. R. and K. Hodivala-Dilke. The role of cell adhesion pathways in angiogenesis. *The International Journal of Biochemistry & Cell Biology* **41** (2009) 521 – 530.

Ravi, R. Mookerjee, B. Bhujwalla, Z. M. Sutter, C. H. Artemov, D. Zeng, Q. Dillehay, L. E. Madan, A. Semenza, G. L and A. Bedi. Regulation of tumour angiogenesis by p53-induced degradation of hypoxia-inducible factor 1α. *Genes & Development* **14** (2000) 34 – 44.

Rea, V. E. A. Rossi, F. W. De Paulis, A. Ragno, P. Selleri, C and N. Montuoril. 67 kDa laminin receptor: structure, function and role in cancer and infection. *Le Infezioni in Medicina* **2** (2012) 8 – 12.

Rehman, A. O. and C. Wang. Notch signalling in the regulation of tumour angiogenesis. *Trends in Cell Biology* **16** (2006) 293 – 300.

Ribatti, D. Nico, B. Crivellato, E. Roccaro, A. M and A. Vacca. The history of the angiogenic switch concept. *Leukemia* **21** (2007) 44 – 52.

Ribatti, D. Endogenous inhibitors of angiogenesis. A historical review. *Leukemia Research* **33** (2009) 638 – 644.

Riley, L. B and D. C Desai. The molecularbasis of cancer and the development of targeted therapy. *Surgical Clinics of North America* **89** (2009) 1 – 15.

Riss, T. L. Moravec, R. A. and A. L. Niles. Cell viability assays. (2013). Available from: http://www.ncbi.nlm.nih.gov/books/NBK144065.

Ross, J. S. Gray, K. Gray, G. S. Worland, P. J and M. Rolfe. Anticancer antibodies. *American Journal of Clinical Pathology* **119** (2003) 472 – 485.

Ross, J. S. Schenkein, D. P. Pietrusko, R. Rolfe, M. Linette, G. P. Stec, J. Stagliano, N. E. Ginsburg, G. S. Symmans, W. F. Pusztai, L and G. N. Hortobagyi. Targeted therapies for cancer. *American Journal of Clinical Pathology* **122** (2004) 598 – 609.

Sagar, S. M. Yance, M. H and R. K. Wong. Natural health products that inhibit angiogenesis: a potential source for investigational new agents to treat cancer—Part 1. *Current Oncology* **13** (2006) 14 – 26.

Salvadori, D. M. F and G. N. da Silva. Genetic instability in normal-appearing and tumour urothelium cells and the role of the TP53 gene in the toxicogenomic effects of antineoplastic drugs, advances in the scientific evaluation of bladder cancer and molecular basis for diagnosis and treatment. (2003). Available from: http://www.intechopen.com.

Saraste, A and K. Pulkki. Morphological and biochemical hallmarks of apoptosis. Cardiovascular Research 45 (2000) 528 – 537.

Sarkar, S. Horn, G. Moulton, K. Oza, A. Byler, S. Kokolus, S and M. Longacre. Cancer development, progression, and therapy: an epigenetic overview. *International Journal of Molecular Sciences* **14** (2013) 21087 – 21113.

Sato, Y. Molecular mechanism of angiogenesis transcription factors and their therapeutic relevance. *Pharmacology & Therapeutics* **87** (2000) 51 – 60.

Scott, A. M. Wolchok, J. D and L. J. Old. Antibody therapy of cancer. *Nature Reviews Cancer* **12** (2012) 278 – 287.

Seaman, S. Stevens, J. Yang, M. Y. Logsdon, D. Graff-Cherry, C and B. St Croix. Genes that distinguish physiological and pathological angiogenesis. *Cancer Cell* **11** (2007) 539 – 554.

Semenza, G.L. HIF-1: using two hands to flip the angiogenic switch. *Cancer and Metastasis Reviews* **19** (2000) 5 9– 65.

Serini, G. Valdembri, D and F. Bussolino. Integrins and angiogenesis: a sticky business. *Experimental Cell Research* **312** (2006) 651 – 658.

Shchors, K and G. Evan. Tumour angiogenesis: cause or consequence of cancer. *Cancer Res*earch **67** (2007) 7059 – 7061.

Singh, S. Sadanandam, A and R. K. Singh. Chemokines in tumour angiogenesis and metastasis. *Cancer Metastasis Reviews* **26** (2007) 453 – 467.

Skinner, H. D. Zheng, J. Z. Fang, J. Agani, F and B-H Jiang. Vascular Endothelial Growth Factor transcriptional activation is mediated by hypoxia-inducible factor 1α, HDM2, and p70S6K1 in response to phosphatidylinositol 3-kinase/Akt signalling. *The Journal of Biological Chemistry* **279** (2004) 45643 – 45651.

Slack-Davis, J. K. Martin, K. H. Tilghman, R. W. Iwanicki, M. Ung, E. J. Autry, C. Luzzio, M.J. Cooper, B. Kath, J.C. Roberts, W. G and J. T. Parsons. Cellular characterization of a novel focal adhesion kinase inhibitor. *The Journal of Biological Chemistry* **282** (2007) 14845 – 14852.

Sliva, D. Rizzo, M. T and D. English. Phosphatidylinositol 3-Kinase and NF-kB regulate motility of invasive MDA-MB-231 human breast cancer cells by the secretion of urokinase-type plasminogen activator. *The Journal Of Biological Chemistry* **277** (2002) 3150 – 3157.

Smith, T. G. Robbins, P. A. and P. J. Ratcliffe, The human side of hypoxia-inducible factor. *British Journal of Haematology* **141** (2008) 325 – 334.

Sottile, J. Regulation of angiogenesis by extracellular matrix. *Biochimica et Acta* **1654** (2004) 13 – 22.

Sparmann, A. and D. Bar-Sagi, Ras-induced interleukin-8 expression plays a critical role in tumour growth and angiogenesis. *Cancer Cell* **6** (2004) 447 – 457.

Srinivas, P. R. Verma, M. Zhao, Y. and S. Srivastava. Proteomics for cancer biomarker discovery. *Clinical Chemistry* **48** (2002) 1160 – 1169.

Stein, C. J and G. A Colditz. Modifiable risk factors for cancer. *British Journal of Cancer* **90** (2004) 299 – 303.

Stein, R. C. Prospects for phosphoinositide 3-kinase inhibition as a cancer treatment. *Endocrine-Related Cancer* **8** (2001) 237 – 248.

Sugimachi, K. Tanaka, S. Terashi, T. Taguchi, K. Rikimaru, T and K. Sugimachi. The mechanisms of angiogenesis in hepatocellular carcinoma: Angiogenic switch during tumour progression. *Liver Cancer* **131** (2002) 135 – 141.

Sun, H. Oudit, G. Y. Ramirez, R.J. Constatini, D. and P. H. Backx. The phosphoinositide 3-kinase inhibitor LY294,002 enhances cardiac myocyte contractility via a direct inhibition of $I_{k\ slow}$ currents. *Cardiovascular Research* **62** (2004) 509 – 520.

Tahergorabi, Z and M. Khazaei. A review on angiogenesis and its assays. *Iranian Journal of Basic Medical Sciences* **15** (2012) 1110 – 1126.

Takac, T. Pechan, T. Samajova, O and J. Samaj. Vesicular trafficking and stress response coupled to PI3K inhibition by LY294,002 as revealed by proteomic and cell biological analysis. *Journal of Proteome Research* **12** (2013) 4435 – 4448.

Takeda, M. Ito, W. Tanabe, M. Ueki, S. Kihara, J. Kato, H. Tanigai, T. Kayaba, H. Sasaki, T. and J. Chihara. The pathophysiology roles of PI3Ks and therapeutic potential of selective inhibitors in allergic inflammation. *International Archives of Allergy and Immunology* **152** (2010) 90 – 95.

Talmadge, J. E and I. J. Fidler. The biology of cancer metastasis: historical perspective. *Cancer Research* **70** (2010) 5649 – 5669.

Terman, B. I and K. V. Stoletov. VEGF and tumour angiogenesis. *The Einstein Journal of Biology and Medicine* **18** (2001) 59 – 66.

Tokunaga, E. Kimura, Y. Mashino, K. Oki, E. Kataoka, A. Ohno, S. Morita, M. Kakeji, Y. Baba, H. Maehara, Y. Tokunaga, E. Kimura, Y. Mashino, K. Oki, E. Kataoka, A. Ohno, S. Morita, M. Kakeji, Y. Baba, H and Y. Maehara. Activation of PI3K/Akt signalling and hormone resistance in breast cancer. *Breast Cancer* **13** (2006) 137 – 144.

Tonini, T. Rossi, F and P. P. Claudio. Molecular basis of angiogenesis and cancer. *Oncogene* **22** (2003) 6549 – 6556.

Valastyan, S and R. A. Weinberg. Tumour Metastasis: Molecular Insights and Evolving Paradigms. *Cell* **147** (2011) 275 – 292.

Valster, A. Tran, N.L. Nakada, M. Berens, M.E. Chan, A.Y and M. Symons. Cell migration and invasion assays. *Methods* **37** (2005) 208 – 215.

van Meerloo, J. Kaspers, G.J. and Cloos, J. Cell sensitivity assays: the MTT assay**.** *Methods in Molecular Biology* **731** (2011) 237 – 245.

van Zijl, F. Krupitza, G and W. Mikulits. Initial steps of metastasis: cell invasion and endothelial transmigration. *Mutation Research* **728** (2011) 23 – 34.

Vara, J.A.F. Casado, E. de Castro, J. Cejas, P. Belda-Iniesta, C and M. Gonzalez-Baron. PI3K/Akt signalling pathway and cancer. *Cancer Treatment Reviews* **30** (2004) 193 – 204.

Vaupel, P. The role of hypoxia-induced factors in tumour progression. *The Oncologist* **9** (2004) 10 – 17.

Vemulapalli, S. Mita, A. Alvarado, Y. Sankhala, K and M. Mita. The emerging role of mammalian target of rapamycin inhibitors in the treatment of sarcomas. *Target Oncology* **6** (2011) 29 – 39.

Vincent, T.L and R.A. Gatenby. An evolutionary model for initiation, promotion, and progression in carcinogenesis. *International Journal of Oncology* **32** (2008) 729 – 737.

Wagsater, D. Lofgren, S. Hugander, A. Dienus, O and J. Dimberg. Analysis of single nucleotide polymorphism in the promoter and protein expression of the chemokine eotaxin-1 in colorectal cancer patients. *World Journal of Surgical Oncology* **5** (2007) 1 – 12.

Wang, J and M.J. Lenardo. Roles of caspases in apoptosis, development, and cytokine maturation revealed by homozygous gene deficiencies. *Journal of Cell Science* **113** (2000) 753 – 757.

Ward, S. Sotsios, Y. Dowden, J. Bruce, I. and P. Finan. Therapeutic potential of phosphoinositide 3-kinase inhibitors. *Chemistry & Biology* **10** (2003) 207 – 213.

Westhoff, M. A. Serrels, B. Fincham, V. J. Frame, M. C and N. O. Carragher. Src-mediated phosphorylation of Focal Adhesion Kinase couples actin and adhesion dynamics to survival signaling. *Molecular and Cellular Biology* **24** (2004) 8113 – 8133.

Widakowich, C. de Castro Jr, G. de Azambuja, E. Dinh, P and A Awada. Review: side effects of approved molecular targeted therapies in solid cancers. *The Oncologist* **12** (2007) 1443 – 1455.

Wilson, J. and F. Balkwill. The role of cytokines in the epithelial cancer microenvironment. *Cancer Biology* **12** (2002) 113 – 120.

Wirtz, D. Konstantopoulos, K and P. C. Searson. The physics of cancer: the role of physical interactions and mechanical forces in metastasis. *Nature Reviews* **11** (2011) 512 – 522.

Wong, C. H. Iskandar, K. B. Yadav, S. K. Hirpara1, J. L. Loh, T and S. Pervaiz. Simultaneous induction of non-canonical autophagy and apoptosis in cancer cells by ROS-de pendent ERK and JNK activation. *Public Library of Science* **5** (2010) 1 – 12.

Wong, K-K. Engelman, J. A. and L. C. Cantley. Targeting the PI3K signalling pathway in cancer. *Current Opinion in Genetics and Development* **20** (2010) 87 – 90.

Wymann, M. P. Zvelebil, M. and M. Laffargue. Phosphoinositide 3-Kinase signalling – which way to target. *Trends in Pharmacological Sciences* **24** (2003) 366 – 376.

Xing, C. Zhu, B. Liu, H. Yao1, H and L. Zhang. Class I phosphatidylinositol 3-kinase inhibitor LY294002 activates autophagy and induces apoptosis through p53 pathway in gastric cancer cell line SGC7901. *Acta Biochimica et Biophysica Sinica* **40** (2008) 194 – 201.

Xue, C. Gudkov, A. Haber, M and M.D. Norris. Small molecule drugs and targeted therapies for neuroblastoma. *Intechopen* **10** (2012) 299 – 325.

Yamaguchi, H. Wyckoff, J and J. Condeelis. Cell migration in tumours. *Current Opinion in Cell Biology* **17** (2005) 559 – 564.

Yamaguchi, K. Ogawa, K. Katsube, T. Shimao, K. Konno, S. Shimakawa, T. Yoshimatsu, K. Naritaka, Y. Yagawa, H and K Hirose. Platelet Factor 4 gene transfection into tumour cells inhibits angiogenesis, tumour growth and metastasis**.** *Anticancer Research* **25** (2005) 847 – 852.

Yang, C. Kaushal, V. Shah, S. V and G. P. Kaushal. Autophagy is associated with apoptosis in cisplatin injury to renal tubular epithelial cells. *American Journal of Physiology* **294** (2008) 777 – 787.

Yokota, J. Tumour progression and metastasis. *Carcinogenesis* **21** (2000) 497 – 503.

Zetter, B. R. Angiogenesis and tumour metastasis. *Annual Review of Medicine* **49** (1998) 407 – 424.

Zhang, Q. Chen, G. Liu, X and Q. Qian. Monoclonal antibodies as therapeutic agents in oncology and antibody gene therapy. *Cell Research* **17** (2007) 89 – 99.

Zhao, J and J-L Guan. Signal transduction by focal adhesion kinase in cancer. *Cancer Metastasis Reviews* **49** (2009) 28 – 35.

Zhao, X and J-L Guan. Focal adhesion kinase and its signalling pathways in cell migration and angiogenesis. *Advanced Drug Delivery Reviews* **18** (2011) 610 – 615.

Zhou, W. Guo, S and RR Gonzalez-Perez. Leptin pro-angiogenic signature in breast cancer is linked to IL-1 signalling. *British Journal of Cancer* **104** (2011) 128 – 137.

Ziyad, S and M. L. Iruela-Arispe. Molecular mechanisms of tumour angiogenesis. *Genes & Cancer* **2** (2011) 1085 – 1096.

Susantad, T and D. R. Smith. siRNA-mediated silencing of the 37/67-kDa high affinity laminin receptor in Hep3B cells induces apoptosis. *Cell and Molecular Biology Letters* **13** (2008) 452 – 464.

Khusal, R. Da Costa Dias, B. Moodley, K. Penny, C. Reusch, U. Knackmuss, S. Little, M and S. F. T. Weiss. In vitro inhibition of angiogenesis by antibodies directed against the 37kda/67kda laminin receptor. *Public Library of Science One* **8** (2013) 1 – 7.