Chapter 2

LITERATURE RIVIEW

2.1 CANCER

As incidence of cancer in the developed countries is rising, and its mortality occupies the second rank in the order of death causes, next to heart diseases, cancer therapy is in the focus of common interest. As per the survey in 2002 by agency GLOBOCAN, malignancy is a global problem with its entire consecutive burden.

Cancer causes about 13% of all deaths (WHO, February 2006). Anyone can get cancers irrespective of race, sex, and age. Although cancer risk increases with age but incidence, prevalence and mortality in different gender and age vary by type of cancer.

2.1.1 What is Cancer?

Carcinogenesis is a complex multistep process, which leads to transformation of a normal cell into a cancerous phenotype and uncontrolled multiplication and spread of the same.

Cancer cells manifest four characteristics that distinguish them from normal cells- uncontrolled proliferation, dedifferentiation and loss of function, invasiveness, and metastasis.

Cancer invades its host by exploiting a cell's natural ability to reproduce life. Some precancerous events causes the normal cell cycle to go wrong; checkpoints are bypassed; mitosis is hijacked; and if further repair mechanisms fail, a malignant cell is born. Further cell replication leads to the formation of a tumor. (Wootton , 2006)

2.1.2 Growth of solid tumors:

Tumors are either benign (non-cancerous) or malignant (cancerous). The term solid tumor is used to distinguish between a localized mass of tissue and leukaemia (tumor with fluid properties). Over 85% of human cancers are solid tumors.

The alterations of genes are responsible for transfer of normal cell to cancer cell. There are diverse ways to cause genomic changes which can cause generation of cancer cells.

Generally changes are due to mutation in genes.

The rate of replication of a single cancerous cell will be higher than the other healthy surrounding cells, placing a strain on the nutrient supply and elimination of metabolic waste products. Tumor cells will displace healthy cells until the tumor reaches a diffusion limited maximum size. Tumor cells can not start apoptosis (a type of programmed cell death) without sufficient oxygen, glucose and amino acid; tumor cells will therefore continue dividing. The

cells which are inside the tumor die because of insufficient nutrient supply, producing necrotic core within tumors. In essence, a steady state tumor size forms, as the rate of proliferation is equal to the rate of cell death until a better connection with the circulatory system is created. This diffusion limited maximal size of most tumors is around 2 mm^3. (Grossfeld et al., 2002; Jones and Harris, 1998; Brannon-peppas and Blanchette , 2004).
To grow beyond this size, the tumor must recruit the formation of blood vessels to provide the nutrients necessary to fuel its continued expansion. An illustration of tumor development from a single cell to a diffusion-limited tumor is shown in Figure 1.1.

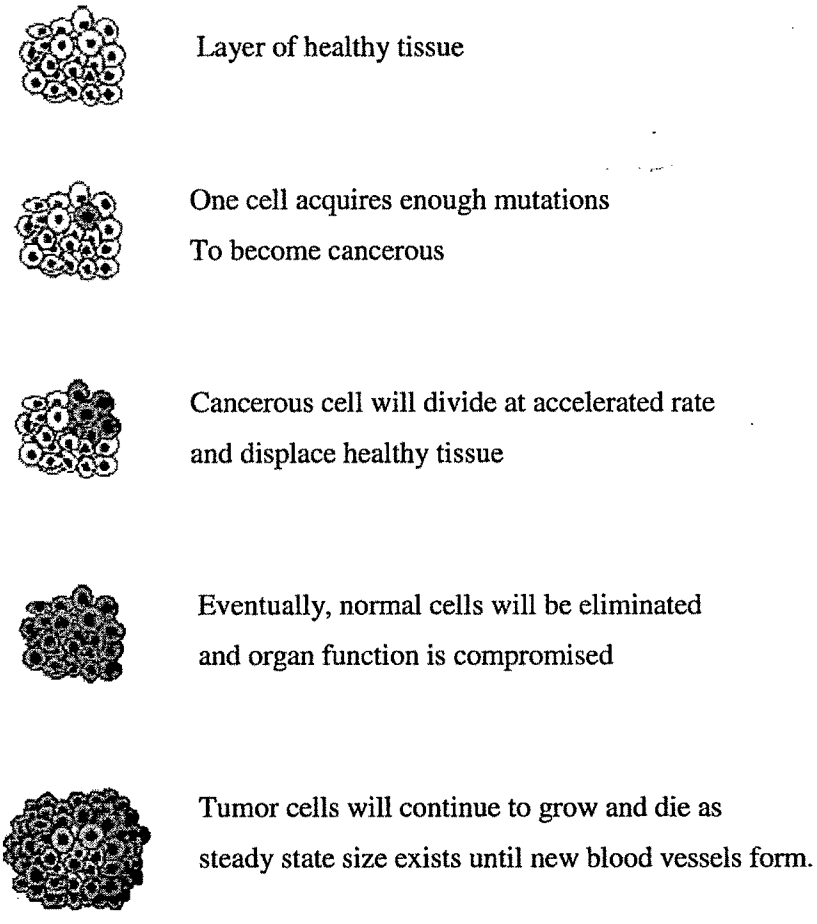

Figure 1.1 Tumor development from initial carcinogenesis to diffusion limited maximal size.

It is thought that there could be numerous tumors at this diffusion limited maximal size through the body. Until the tumor can gain access to the circulation it will remain at this size and the process can take years. Tumor cells are capable of secreting molecules that initiate the angiogenic process. The new vessels will allow the tumor to grow beyond the diffusion-

limited maximal size. Some tumor masses never grow beyond this point, as they are incapable of recruiting new vessels. Thus angiogenesis is a process vital to the continued development and metastasis of a tumor mass.

2.1.3 Cancer therapy:

Conventionally cancer treatment is based on surgery, radiation therapy, chemotherapy, immunotherapy and many more depending on the type of tumor and the stage of the cancer. But this complex approach is far from being satisfactory.

The treatment of cancer with drugs was started by Huggins and Hodgesin 1941, with the discovery that oestrogens palliate prostate cancer. Subsequently Cancer chemotherapy started with the discovery of the cytostatic effect of N-mustard and its derivatives more than five decades ago. Since then a large number of chemotherapeutic drugs are available for cancer treatment.

2.1.3a Surgery:

Surgery is the oldest and most widely used method for the successful treatment of cancer.

This approach involves surgical removal of the tumor, which is usually quick and effective if diagnosed early and effective. Over the years, the improvements in surgical techniques, anaesthesiology, and the control of infection have benefited cancer patients. Mastectomy for breast cancer and prostatectomy for prostate cancer are the examples of surgeries in cancer treatment. Many times surgeries are used in combination with other type of treatments for example radiation may be used before and after surgery.

Surgery, however, has several important disadvantages.

- It is an invasive procedure.
- The removal of a visible tumor does not guarantee elimination of small microscopic tumor extensions (Hellman and Vokes,1996).
- As a result of these extensions, surgeons usually have to excise a substantial amount of surrounding healthy tissue. Such radical surgeries may severely impair the patient's day-to-day functioning or appearance.
- Surgery may not even be a viable option if a tumor encloses a vital organ (Kessel and Dougherty,1983)
- One of the biggest shortcomings of the surgical removal of a tumor relates to potential metastasis, where the cancer cells have already spread (metastasized) throughout the body.

2.1.3b Radiation therapy:

Radiation therapy is a localized treatment whose goal is to deliver a sufficient and uniform dose in the form of ionising radiation to kill cancer cells and shrink tumors. Radiation causes damage in the genes which makes these cells unable to grow and divide. This non-invasive therapy is preferable to surgery in many situations because it can destroy a tumor effectively while inflicting minimal damage to the surrounding healthy tissue (Foye, 1989). In addition, normal tissue can recover from radiation exposure more quickly than tumor cells. X-rays or gamma rays are used to irradiate the region of the body where the cancerous tumor exists. Most commonly, irradiation methods result in genetic damage sufficient to either kill cells directly or indirectly by inducing cellular suicide (apoptosis) (Fisher, 1994). Radiation treatments have an advantage over surgery in that the small microscopic extensions of cancerous tissue surrounding a tumor that might have been missed otherwise are destroyed. Radiation therapy also suffers from some shortcomings.

- Radiation can fail to completely eradicate all the cancer cells of a tumor, which is also a concern regarding the surgical removal of tumors (Foye, 1989).
- Radiation therapy is essentially alocalized event. Consequently, the procedure cannot treat widespread metastases that will eventually develop into tumors at distant sites.
- Finally, repeated or prolonged exposureto all forms of ionizing radiation has been demonstrated to cause cancer under the "right" conditions.
- Normal body tissues vary in their response to radiation. Some cells in normal tissues divide more rapidly may be affected by radiations and also may cause damage to the nearby tissues, organs and bones.
- Some of the serious side effects of the radiation therapy depending on the part of the body exposed to radiation are; memory loss, lower sexual desire, permanent loss of sperm production, fibrosis of lung, swollen and inflamed esophagus, stomach etc.

2.1.3c Chemotherapy

Surgery and radiation do not provide effective treatments for cancer that has already spread to distant parts of the body. In such cases, the administration of systemic anticancer agents (chemotherapy) may be used. Compared to surgery and radiotherapy, chemotherapy is not limited by metastasis as much as by the total mass of the tumor (Berkow,1977).

The term chemotherapy means destruction of cancerous cells with anticancer drugs.

Anticancer drugs may be: cell-cycle active, phase-specific agents for example antimetabolites and vinca alkalois Or phase non-specific which can injure DNA at any phase of cell cycle but

appear to block the check points before the cell division for example alkylating agents and antitumor antibiotics or hormones. (Satoskar et al., 1997). Many times chemotherapy uses various anticancer drugs regimens together called combination chemotherapy, because some drugs work better in combination than alone. At some point all cancer patients have to undergo chemotherapy during the treatment. Chemotherapy agents mostly used now days are anthracyclines, camptothecins, platinates, and taxanes.
(http://www.ctiseattle.com/about.htm.)
Unfortunately, there are significant limitations for chemotherapy in cancer treatment.

- The cytotoxic drugs, act by affecting the process of cell division. Hence, they also affect all rapidly dividing normal cells, leading to common toxic effects such as: Bonemarrow depression, loss of hair, damage to GI mucosa and ulceration, impaired wound healing, damage to gonads, growth inhibition in children and teratogenicity.
- The principal limitations of chemotherapy in treating cancer are its side effects and systemic toxicity and lack of specificity of chemotherapeutic agent. Also the inadequate delivery of drugs to the tumor cells would result in residual tumor cells, which in turn would lead to regrowth of tumors and result even in development of resistant cells.
- Some tumors can even develop resistance to multiple drugs after only one drug has been administered to the patient.

The degree of change in the patient's quality of life and eventual life expectancy is directly related to the targeting ability of the treatment. But unfortunately the above treatment modalities are frequently insufficient to eliminate viable cancer cells without exceeding the limits of toxicity to normal tissue.
Current research areas include development of carriers to allow alternative dosing routes, new therapeutic targets such as blood vessels fuelling tumor growth and targeted therapeutics that are more specific in their activity (Shenoy et al., 2005; Brannon-peppas and Blanchette, 2004).
The development of antitumor drug therapy along with new delivery systems is the extensively explored area in cancer research. With the revolutionary discoveries in the molecular biology and better understanding of biology of cancer, the specific targets can be identified in tumor cells, the function of which are necessary prerequisites for the replication of the same (Eckhardt , 2002).

The study of cancer at molecular level better enables us to design drugs to halt their proliferation and spread. This new approach of targeting the mechanisms by which cancer cells prosper has been called, appropriately, a mechanism-based approach. One of the most promising new approaches to cancer chemotherapy is the use of angiogenesis inhibitors. Angiogenesis is a prerequisite process to the growth and metastasis of tumor. A detailed account on the angiogenesis and angiogenesis inhibitors is presented in the next section.

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2.2 TUMOR ANGIOGENESIS

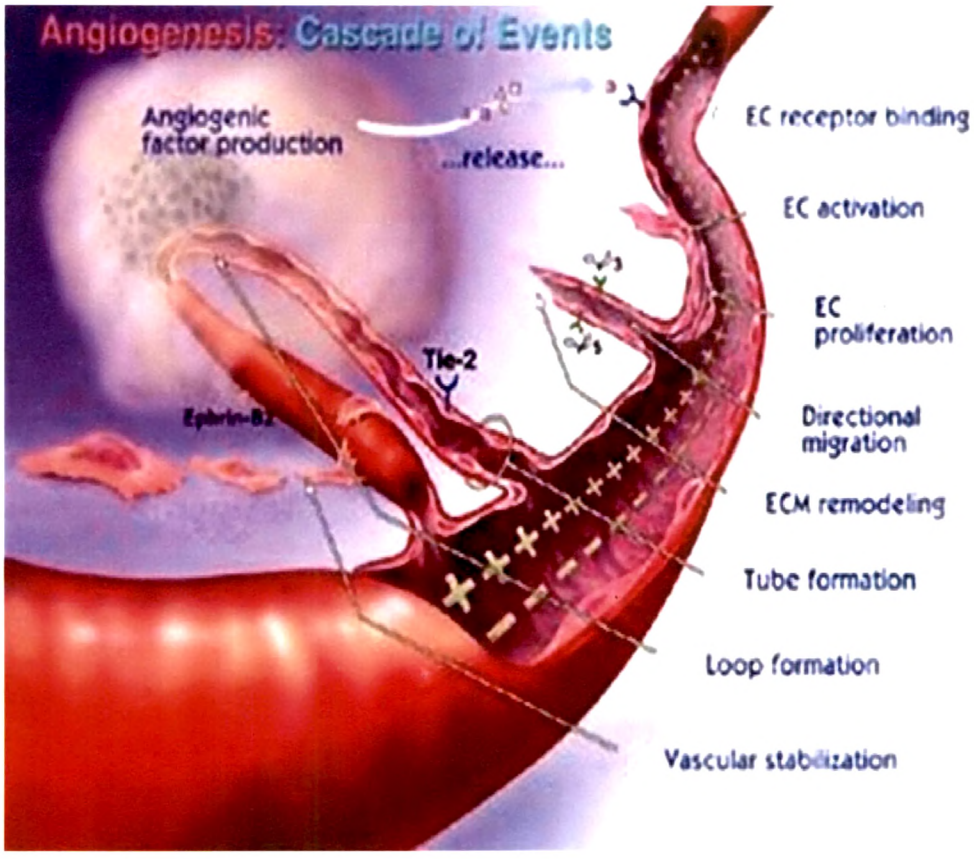


Figure 2.1: Process of Angiogenesis

2.2.1 Angiogenesis:

Nowadays Angiogenesis process is an intensely researched topic. Angiogenesis is the formation of new blood vessels from pre-existing ones. (Severin et al., 2002) It is needed anywhere new tissue is growing. In adults, it occurs during physiological events like wound healing, ovulation, menstruation and pregnancy. Angiogenesis is also encountered in patients recovering from heart attacks or strokes. Nonetheless, it is true that angiogenesis can also exacerbate several diseases currently termed as angiogenesis-dependent diseases.

Approximately 20 angiogenesis-dependent diseases have been identified so far. The formation of new blood vessels is observed during hemangiomas, hypertrophic scarring, keloids, warts, periodontitis, sclerodermia, neovascularization of corneal grafts and neovascular glaucoma. Abnormal angiogenesis which is also referred as pathological

angiogenesis takes place in the case of specific disease states, including rheumatoid arthritis, psoriasis, atherosclerosis, tumour growth and metastasis. (Klagsbrun and Moses, 1999).

Under physiologic conditions, the vascular endothelium is a quiescent tissue with very low cell division rate i.e. turnover times ranging in hundreds of days. During angiogenesis, endothelial cells emerge from their quiescent state and can proliferate rapidly with turnover times of 4 to 5 days (Folkman, 1997).

2.2.2 History: Angiogenesis

In the early 1960, Folkman and Becker, while developing blood substitutes, perfused rabbit thyroid glands with haemoglobin solution and then inserted tiny melanoma implants to learn whether the blood substitute supported tumour growth. Unexpectedly, all implants reached a few millimetres in size and then abruptly stopped growing. When replaced subcutaneously in their original mouse host, the implants developed new blood vessels and rapidly grew to a large size (Folkman et al., 1962). This work was supported by a study showing that tumours implanted in the anterior chamber of the eye grow only as large as the head of a pin, but after being pushed back into the vascularised iris they grow rapidly. Thus Folkman proposed that some diffusible message is released from tumour to nearby endothelial cells that are then "switched" from their resting state to divide rapidly and form new sprouts. (Folkman, 1971) Soon after this, tumour angiogenesis factor was isolated from human and animal tumours (Folkman et al, 1971)

The first factor isolated was basic fibroblast growth factor (bFGF) (Shing, et al., 1984), which is a potent mitogen for endothelium. The second factor to be isolated was vascular endothelial growth factor (VEGF) (Senger et al., 1983). VEGF is perhaps the most potent endothelial chemoattractant, stimulating endothelial cells to migrate in a directional manner. VEGF was initially discovered as a potent vascular permeability factor (VPF), which stimulates blood vessels to leak, a common finding in tumours. Later in 1989, Dr. Napolene Ferra identified and isolated VEGF. Subsequently, a number of other factors have been identified to stimulate angiogenesis, and these factors, consisting of both small molecules and peptides, interacts with either tyrosine kinase receptors or G-protein-coupled receptors (Tachibana et al., 1998; Furness et al., 2005).

Thus angiogenesis has proven to be the result of numerous interactions between regulators, mediators and stimulatory molecules. These molecules regulate the proliferative and invasive activity of the endothelial cells that line blood vessels. (Brannon-Peppas and Blanchette., 2004)

2.2.3 Tumour growth is angiogenesis dependant

Long standing research shows that tumours cannot grow past about 1-2 mm without their own blood vessels. Angiogenesis and tumour progression are very closely linked with each other. Tumour cells are dependent on angiogenesis because their growth and expansion require oxygen and nutrients, which are made available through the angiogenic vasculature. Tumour growth is often a multi-step process that starts with the loss of control of cell proliferation. The cancerous cell then begins to divide rapidly, resulting in a microscopically small, spheroid tumour: an *in situ* carcinoma (Folkman, 1995). As the tumour mass grows, the cells will find themselves further and further away from the nearest capillary. Finally, the tumour stops growing and reaches a steady state, in which the number of proliferating cells counterbalances the number of dying cells. The restriction in size is caused by the lack of nutrients and oxygen (Gasparini, 1999). *In situ* carcinomas may remain dormant and undetected for many years, and metastases are rarely associated with these small (2–3 mm^3, avascular tumours (Folkman, 1995). Several months or years later, an *in situ* tumour may switch to the angiogenic phenotype, induce the formation of new capillaries, and start to invade the surrounding tissue.

2.2.4 Angiogenic Switch

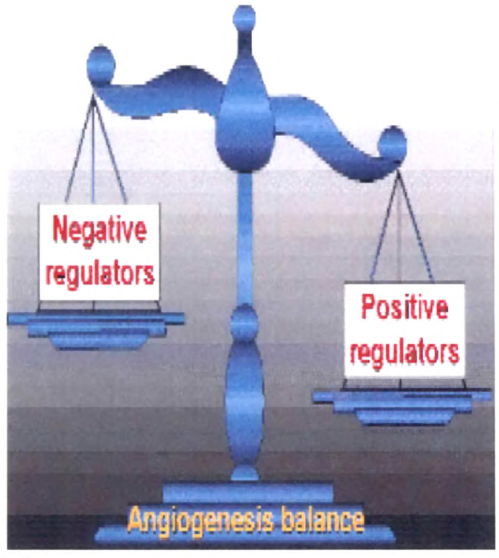
Angiogenesis is tightly controlled through a balance of pro and antiangiogenic factors. (Table 2.1, Kerbel, 2000) In healthy tissue, the balance weighs heavily to the antiangiogenic side. Tumour cells can cause a shift in the balance toward proangiogenesis.

Anti-Angiogenesis

Thrombospondin-1

Angiostatin

Endostatin



Pro-angiogenesis

Vasculoendothelial Growth Factor (VEGF)

Acidic And Basic Fibroblast Growth Factor (aFGF/bFGF)

Angiogenin

Epidermal Growth Factor (EGF)

Figure 2.2: Angiogenesis balance

Endogenous angiogenic factors.

Pro-angiogenic factors	Antiangiogenic factors
Growth factors	Proteolytic fragments
VEGF	Angiostatin
FGF (acidic & basic)	Endostatin
Hepatocyte growth factor	Serpin antithrombin
Platelet-derived growth factor	Canstatin
EGF	PEX
Granulocyte colony-stimulating factor	Prolactin (16 kD)
Tumour necrosis factor α	Restin
Cytokines	Tumstatin
Interleukin-1, -6 and -8	Arresten
Enzymes	Vasostatin
Cathepsin	Kringle 1–5
Gelatinase A,B	Fibronectin fragments
Stromelysin	Cytokines and Chemokines
Small adhesion molecules	Interleukin-1, -4, -10, -12 and -18
$\alpha_v\beta_3$ integrin	Interferon-α, -β, -γ
Metal ions	EMAP-II
Copper	gro-β
Others	IP-10
Angiostatin-2	Monokine induced by interferon-γ
Angiopoietin-1	Platelet factor 4
Angiotropin	Soluble receptors
Angiogenin	Soluble FGFR-1
Adrenomedullin	Soluble VEGFR-1
Erythropoeitin	Collagenase inhibitor
Endothelin	TIMP-1, -2, -3 and -4
Hypoxia	Vitamins
Midkine	1,25 (OH) vitamin D_3
Nitric oxide synthase	Retinoic acid
Prostaglandin F	Tumour suppressor genes
Pleiotropin	p16
Platelet activating factor	p53
Plasminogen activator inhibitor 1	Other inhibitors
Thymidine phosphorylase	Angiopoietin
Thrombopoietin	Angiotensin
Urokinase tissue plasminogen activator	Angiotensin-2-receptor
	Canecin
	Meth-1, -2
	2-Methoxy oestradiol
	Osteopontin cleavage product
	Pigment epithelium derived factor
	Prostate specific antigen
	Protamine
	Thrombospondin-1, -2
	Transforming growth factor-β1
	Troponin I

EMAP-11, endothelial monocyte-activating polypeptide II; IP-10, interferon-γ-inducible protein 10; TIMP, tissue inhibitor of MMPs.

Table 2.1: Endogenous angiogenesis factors (adapted from Kerbel, 2000)

Several observations led to the development of the angiogenic switch concept. First, in a transgenic model of cancer, mice engineered to develop tumours in the islet cells of the pancreas were noted to have high levels of the angiogenic stimulator VEGF prior to the development of malignant tumours. Despite this, the density of vessels only increased with increasing tumour growth, leading to the development of the concept of a balance between endogenous angiogenesis stimulators and inhibitors. Second, (Arbiser et al., 1997) demonstrated that introduction of oncogenic ras, which is mutated in up to 40% of human cancers, causes upregulation of proangiogenic factors such as VEGF and matrix metalloproteinases, and downregulation of the endogenous tissue inhibitor of matrix metalloproteinases. Finally, loss of tumour suppressor genes such as p53 was noted to cause

upregulation of VEGF, and conversely, reintroduction of tumour suppressor genes result in downregulation of VEGF. Thus, while early tumours may upregulate angiogenic factors such as VEGF, rapid tumour growth does not occur until the relative proportion of angiogenesis stimulators exceeds the production of angiogenesis inhibitors. (Furness et al., 2005)

The "angiogenic switch" depends on a net balance of positive and negative angiogenic factors in the tumour, as depicted in the above fig. and table. Thus, the angiogenic phenotype may result from the production of growth factors, such as FGF-2 and VEGF, by tumour cells and/or the down-regulation of negative modulators, like TSP-1, in tissues with a quiescent vasculature (Pepper, 1997). In both normal and pathological angiogenesis, hypoxia is the main force initiating the angiogenic process. In a tumour, the angiogenic phenotype can be triggered by hypoxia resulting from the increasing distance of the growing tumour cells to the capillaries or from the inefficiency of the newly formed vessels. Hypoxia induces the expression of VEGF and its receptor via HIF-1a (Carmeliet et al., 1998; Jiang et al., 1997) and is also an attractant for macrophages. Also, several oncogenes such as v-*ras,* K-*ras,* v-*raf, src, fos* and v-*yes* (Jiang et al.,1997; Kerbel et al.,1998; Okada et al., 1998) induce the up-regulation of angiogenic factors like VEGF and increase the production of cytokines and proteolytic enzymes. Moreover oncogene products may act directly as angiogenic factors. In contrast, the tumour suppressor p53 has been found to cause degradation of HIF-1α (Ravi et al., 2000), inhibition of VEGF production (Mukhopadhyay et al., 1995), and stimulation of the inhibitor TSP-1(Dameron et al., 1994).

2.2.5 The Process of Angiogenesis:

Tumor blood vessels can form from pre-existing vessels by angiogenic sprouting or by intussusceptive growth. During angiogenic sprouting, the process that has been most extensively studied, endothelial cell activation, migration and proliferation take place in a highly regulated manner. Blood vessel maturation occurs *via* migration and support of pericytes and local changes in growth factor composition as elaborated below. (Molema, 2005).

Anatomically speaking, a typical blood vessel is formed of an external envelope called basement membrane and composed mainly of collagen fibers. This envelope is lined on the inside by a monolayer of endothelial cells, the main cellular component of the blood vessel. Red blood cells, immune and inflammatory, "white blood cells" and different soluble nutrients circulate into it. Various triggering events (signal source) can induce the process of

angiogenesis. During inflammation or hypoxia, pro-angiogenic factors (growth factors, cytokines) favouring the formation of blood vessels are conveyed to neighboring established blood vessels (step 1). One of the first cellular events observed during the angiogenesis process is the extravasion and migration of inflammatory cells toward the signal source. Through the secretion of additional proangiogenenic factors, inflammatory cells amplify the signal (step II). Endothelial cells' response to the pro-angiogenic signal is the secretion of

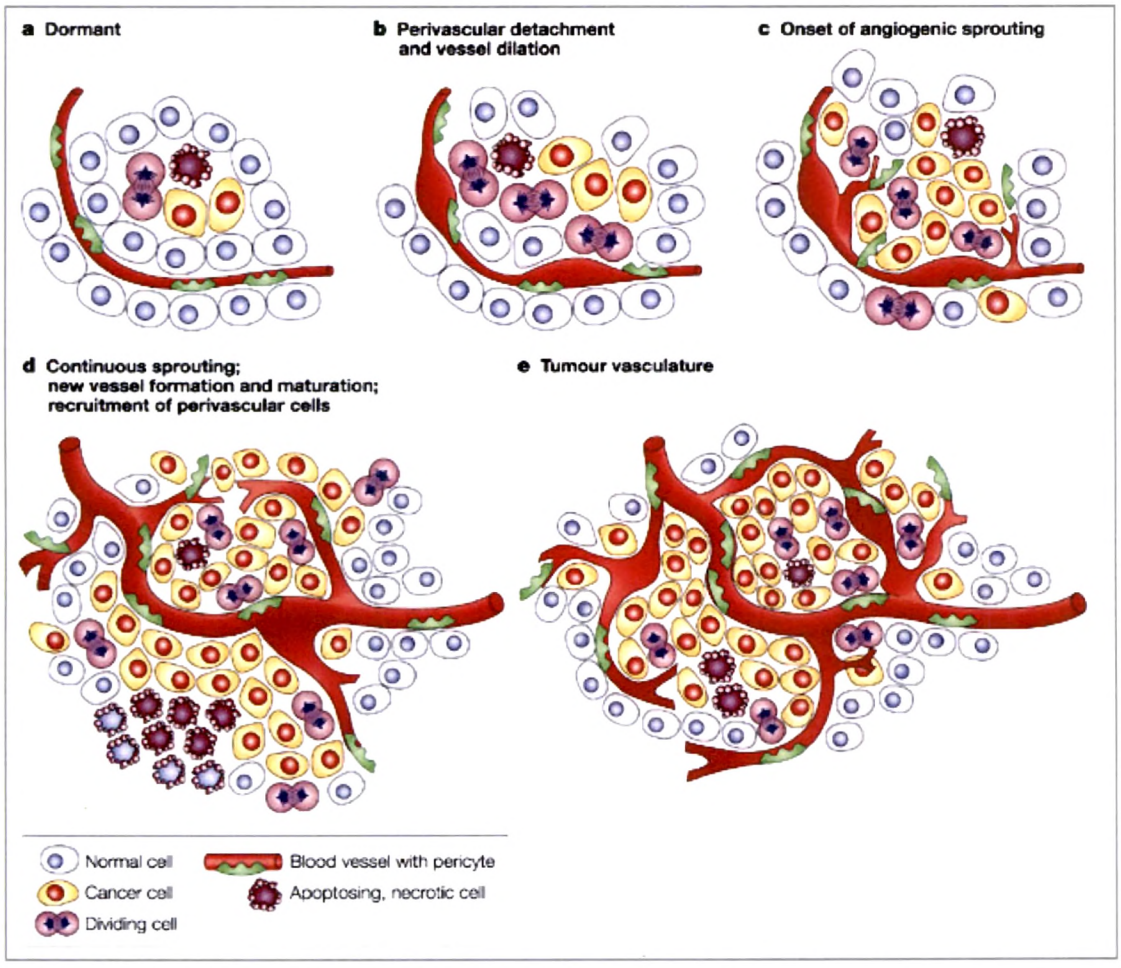


Figure 2.3: The angiogenic switch: (**a**) Almost all tumours start growing as avascular nodules (dormant) until they reach a steady-state level of proliferating and apoptotic cells. The angiogenic switch starts when tumour needs the oxygen and nutrients in order to grow. (**b**) The switch begins with vessel dilation and perivascular detachment, then (**c**) angiogenic sprouting starts, (**d**) the new vessel are formed and start to mature; the recruitment of perivascular cells takes place, (**e**) the newly formed blood vessels will specifically feed hypoxic and necrotic areas of the tumour to provide it with essential nutrients and oxygen so the tumour can grow (Adapted from Bergers and Benjaminl, 2003).

specialized enzymes called matrix metalloproteinases that digest the collagen fibers present in the basement membrane. This creates a breach through which proliferating endothelial cells can migrate in the direction of the signal source (step III). Along their path, endothelial cells produce an extracellular matrix that is assembled into an organized basement membrane, leading to the formation of a functional new blood vessel (step IV). If the signal comes from tumour cells, the angiogenesis process may result in the vascularization of the tumour, thereby promoting tumour growth.

2.2.6 Tumour angiogenesis: molecular mechanism

Different cells and stimulating factors are involved in angiogenesis. Some of the cells engaged are the endothelial cells (EC), lymphocytes, macrophages, and mast cells. Vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) are two of the major among many factors involved in this process. These cells and stimulating factors play different and important role during Tumour angiogenesis. This process includes two major phases, namely the **activation phase** and the **formation phase**. Before describing these two phases, it is very important to know what triggers Angiogenesis.

As mentioned above, tumour cells require nutrients and oxygen to overcome hypoxia and starvation. When a condition such as hypoxia is present in the tumour tissue, the tumour cells receive the signal and promote the angiogenic switch and induce angiogenesis (Risau, 1997). In the case of Hypoxia, the signal is mediated by hypoxia inducible factor-1 (HIF-1).

HIF-1 binds to hypoxia-response elements (HREs) and activates a number of hypoxia-response genes such as VEGF. Thus hypoxia upregulates the expression of angiogenic factors, like VEGF. Additionally, the tumour cells export FGF even though until now, no signal for its secretion is known. VEGF and FGF bind to their receptors respectively VEGFR (1-3) and FGFR on the endothelial cells. Both, VEGF and FGF activate signal transduction pathways, activating in this way the Endothelial cells (Figure 2.4). At this time point the first phase of tumour angiogenesis starts.

In the first phase, the **activation phase**, the adventitial cells and pericytes are retracted, while the basal membrane of the pre-existing vessels is degraded by proteases, for example by members of the matrix metalloproteinase family (MMP) (Figure 2.4). MMPs are produced by the activated endothelial cells. Since the basement membrane barrier is disrupted, the endothelial cells, which cover the internal wall of a blood vessel, are able to migrate from pre-existing vessels towards the angiogenic stimuli and proliferate (Bergers and Benjamin, 2003). The migration of the endothelial cells (EC) is based on cell-extracellular interaction

that is mediated by vascular cell-adhesion molecules for example by integrin αvβ3. Research has shown that this molecule that mediates cell adhesion plays an important role in angiogenesis.

During the **formation phase**, the endothelial cells, after migrating, are structured into tubes to form capillary-like structures, these mature into functional capillaries, and then the blood flow is initiated. The **formation phase** is thought to be dependent on E-selectin, which is a transmembrane cell-adhesion glycoprotein. E-selectin mediates endothelial cell-cell interaction. Moreover, mesenchymal cells play a decisive role in the formation of mature blood vessels. These cells express Angiopoetin-1, which binds to Tie-2 receptors expressed on the EC. This binding is thought to help in pericyte recruitment, vessel sprouting and vessel stabilization. Tie receptors (Tie-1 and Tie-2) are tyrosine kinases and their expression follows VEFGR expression. The ligand for Tie-2 is Angiopoetin-1, which upon binding to Tie-2 induces tyrosine phosphorylation of Tie-2. Angiopoetin-1 has showed induction of capillary sprouts formation and EC survival support (Severin et al, 2002).

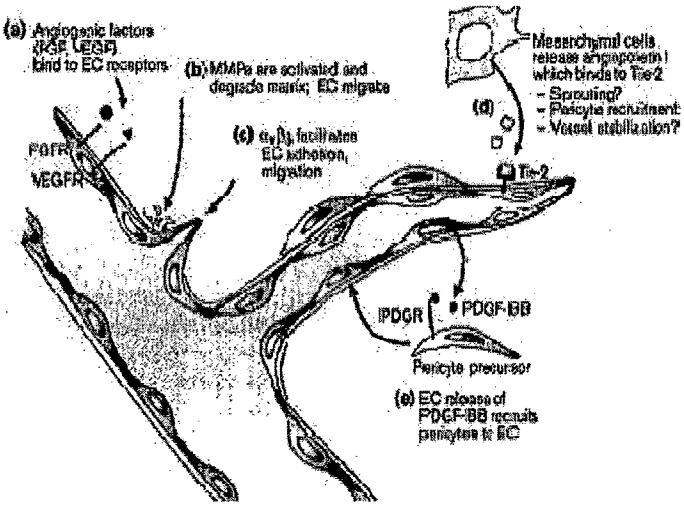


Figure 2.4: The different steps in blood vessel formation

a) VEGF and FGF bind to their receptors on endothelial cells and activate signal transduction pathways.

b) Matrix metalloproteinases (MMPs) are activated and degrade the exctracellular matrix allowing the ECs to migrate out of the pre- existing capillary wall and proliferate. **c)** ECs express Integrin αvβ3 to facilitate their adhesion to the exctracellular matrix and their migration. **d)** Angiopoetin-1 binds to Tie-2 receptors. This binding is thought to stimulate pericyte recruitment, vessel sprouting and vessel stabilization. **e)** PDGF-BB (PDGF isoform) is released by the ECs and acts as a chemoattractant for pericyte precursors. Pericyte precursors join together with the ECs and differentiate into pericytes (Klagsbrun and Moses, 1999).

After recruitment to the scene of act, the mesenchymal cells differentiate into smooth-muscle cell-like pericytes, which cover the vascular tree (Figure 2.4). A special role in pericyte recruitment plays the platelet-derived growth factor (PDGF). PDGF is excreted by the endothelial cell and it functions as a chemoattractant for pericyte precursors, which after associating with endothelial cells differentiate into pericytes (Figure 2.4). The role of pericytes is yet not completely understood, but it is believed that they play a role in stabilizing the newly formed blood vessels.

2.2.7 Angiogenesis regulators: Endogenous factors

Some important endogenous factors which regulates angiogenesis are discussed in this section.

2.2.7.1 Proangiogenic factors:

a) Matrix metalloproteinases (MMPs):

MMPs are a family of secreted or transmembrane proteolytic enzymes that are capable of digesting extracellular matrix and basement membrane components under physiologic conditions. There are currently 25 known MMPs, which differ in their substrate specificity but share a number of common structural and functional similarities. The regulation of MMP activity occurs at various levels including synthesis, secretion, activation and inhibition. MMPs involved in angiogenesis include MMP-1, MMP-2, MMP-9, MMP-12, MMP-19, MMP-26. (Jackson, 2002)

There are three major subgroups of MMPs, identified by their substrate preferences: collagenases degrade fibrillar collagen, stromelysins prefer proteoglycans and glycoproteins as substrates, and gelatinases are particularly potent in degradation of nonfibrillar and denatured collagens (gelatin) (Lynn M. and Matrisian,1997).

In order to preserve the normal tissue structure, a stringent control of local proteolytic activity is ensured by secretion of MMPs along with their inhibitors. However evidence has suggested that this regulation is lost during tumour growth and metastasis. Excessive MMP activity has been detected in colorectal, lung, breast, gastric, cervical, bladder, prostate cancer, and malignant glioblastoma. Moreover, in a numberof these studies, a good correlation was found between the amount of MMPs and the aggressiveness/invasiveness of the tumour (Liekens, 2001).

b) Vascular endothelial growth factor (VEGF):

Vascular endothelial growth factor is a protein 50,000 times more active than histamine in enhancing vascular permeability. VEGF is very similar to vascular permeability factor (VPF),

and it is one of the most potent and important mediators of increased vascular permeability during angiogenesis. (Nagy et al., 1989)

VEGF is produced in response to hypoxic conditions, which are prevalent within tumours.

It is also produced secondarily in response to the production of other growth factors, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), tumour necrosis factor (TNF), and transforming growth factor-beta (TGF-beta) (Liekens, 2001).

VEGF belongs to the VEGF family, which currently consists of six members: VEGF-A (or VEGF), PlGF, VEGF-B, VEGF-C, VEGF-D, and orf virus VEGF (VEGF-E). VEGF is expressed in different tissues, including brain, kidney, liver, and spleen, and by many cell types (Veikkola and Alitalo, 1999). *In vitro*, VEGF stimulates ECM degradation, proliferation, migration, and tube formation of endothelial cells and induces in these cells the expression of MMP-1. *In vivo*, VEGF has been shown to regulate vascular permeability, which is important for the initiation of angiogenesis. Two high-affinity binding sites for VEGF have been identified on vascular endothelium: VEGFR-1 and VEGFR-2. Several studies have indicated that VEGFR-1 and VEGFR-2 have different signal transduction properties (Ferrara, 1999). Interaction of VEGF with VEGFR-2 is critical for VEGF-induced biological responses, whereas the function of VEGFR-1 in VEGF-mediated angiogenesis is still unclear.

c) Angipoitins:

Two other endothelial cell-specific receptors, called Tie-1 and Tie-2, were identified several years ago. Experiments in mice have suggested a role for these receptors in blood vessel maturation. The ligands for Tie-2 have been discovered only recently: angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) both bind Tie-2, but only the binding of Ang-1 results in signal transduction and regulation of blood vessel maturation. Therefore, Ang-2 is a natural antagonist of Ang-1 (Maisonpierre et al., 1997). In a normal adult vessel, Ang-1 is associated with Tie-2 to keep the vessels in a stable state. Up-regulation of Ang-2, by hypoxia or VEGF (Oh H et al,1999), for example in the ovary during corpus luteum formation or by tumour cells, disrupts the interaction between Ang-1 and Tie-2, resulting in destabilization of the vessels. Endothelial cells, which are no longer attached to the pericytes and the ECM, become responsive to angiogenic signals and, in the presence of VEGF, angiogenesis is promoted. The absence of stimulatory signals will cause regression of the vessels (Holash et al., 1999).

d) Basic fibroblast growth factor (FGF-2):

The FGF family consists of at least 19 members. All FGFs are 18- to 30-kDa proteins with high affinity for heparin. FGF-2 was one of the first angiogenic factors to be characterized and has been studied extensively.

FGF-2 exists in isoforms with molecular weight ranging from 18,000 to 24,000. Both low and high molecular weight FGF-2 isoforms show angiogenic activity *in vivo* and induce cell proliferation in cultured endothelial cells. FGF-2 was found to induce tube formation in collagen gels and to modulate integrin expression, gap junction intercellular communication, and VEGF up-regulation *in vitro.* At least four members of high-affinity tyrosine-kinase FGFRs (Johnson and Williams, 1993) have been described. Low-affinity binding sites were identified as proteoglycans, including syndecan and perlecan, containing HS side chains (HSPGs). These HSPGs are found in the ECM, the basement membrane, and the cell surface. It has been suggested that binding of FGF-2 to HSPGs results in protection of FGF-2 from inactivation in the extracellular environment and in storage of FGF-2 in the ECM and basement membrane. Stored FGF-2 can be released by heparitinase and soluble heparin or after ECM breakdown (Johnson and Williams, 1996; Liekens, 2001).

e) Cell adhesion molecules (CAMs):

The processes of cell invasion, migration, and proliferation not only depend on angiogenic enzymes, growth factors, and their receptors, but are also mediated by cell adhesion molecules. To initiate the angiogenic process, endothelial cells have to dissociate from neighboring cells before they can invade the underlying tissue. During invasion and migration, the interaction of the endothelial cells with the ECM is mediated by integrins. Also, the final phases of the angiogenic process, including the construction of capillary loops and lumen formation, involve cell– cell contact and cell–ECM interactions (Bischoff, 1997).

Cell adhesion molecules can be classified into four families depending on their biochemical and structural characteristics. These families include the selectins, the immunoglobulin supergene family, the cadherins, and the integrins.

Integrins are a group of cell adhesion receptors, consisting of non-covalently associated α and β subunits, which can heterodimerize in more than 20 combinations. Endothelial cells thus express several distinct integrins, allowing attachment to a wide variety of ECM proteins. Integrin $\alpha_v\beta_3$ was found to be particularly important during angiogenesis. $\alpha_v\beta_3$ is a receptor for a number of proteins including fibronectin, vitronectin, laminin, fibrinogen, and denatured collagen. In addition, $\alpha_v\beta_3$ has been shown to bind MMP-2, thereby localizing MMP-2-mediated matrix degradation to the endothelial cell surface (Eliceiri and Cheresh, 1999).

$\alpha v\beta_3$ is nearly undetectable on quiescent endothelium, but is highly up-regulated during cytokine- or tumour-induced angiogenesis. In activated endothelium, $\alpha v\beta_3$ suppresses the activity of p53, while increasing the Bcl2:Bax ratio, resulting in an anti-apoptotic effect. Another receptor that has been implicated recently in angiogenesis is integrin $\alpha v\beta_5$. Antibodies directed against $\alpha v\beta 3$ were found to specifically block FGF-2- or TNF- α - induced angiogenesis, whereas antagonists of $\alpha v\beta_5$ blocked VEGF-induced angiogenesis (Friedlander et al., 1995).

Vascular endothelial cadherin or VE-cadherin mediates calcium-dependent homophilic interactions between endothelial cells.

Members of the immunoglobulin superfamily mediate heterophilic cell–cell adhesion. Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are expressed on quiescent endothelium, but are up-regulated after stimulation with TNF-α, IL-1, or IFN-γ (Brooks, 1996). Furthermore, VCAM-1 can induce chemotaxis in endothelial cells *in vitro* and angiogenesis *in vivo*.

Also, members of the selectin family, in particular P-selectin and E-selectin, which promotes adhesion of leukocytes to cytokine-activated vascular endothelium, have been shown to play a role in angiogenesis. E-selectin was found to induce endothelial migration and tube formation *in vitro* and angiogenesis *in vivo* (Koch et al., 1995).

2.2.7.2 Endogenous Antiangiogenic factors:

a) Thrmbospondin-1 (TSP-1):

TSP-1 is considered to be the main physiological inhibitor of angiogenesis, being constitutively produced by normal cells. Its expression is inversely correlated with angiogenesis, i.e. during tumorigenesis, TSP-1 is down-regulated while the angiogenic activity is increased. Accordingly, it was shown that TSP-1 production is regulated by the tumour suppressor p53. Mutation of *p53* results in the loss of TSP-1 production and a switch to the angiogenic phenotype (Iruela-Arispe and Dvorak,1997). Consequently, overexpression of TSP-1 causes a decrease in angiogenesis and inhibition of tumour growth (Streit et al, 1999).

b) Angiostatin and Endostatin:

Angiostatin and endostatin are examples of endogenous inhibitors that are generated by the proteolysis of inactive circulating precursors.

Angiostatin is produced by the proteolytic activity on plasminogen. *In vivo* experiments in mice have shown that angiostatin suppresses the growth of a number of human tumours and their metastases (Cao, 1999). A recent report showed that ATP synthase binds angiostatin,

implying that angiostatin interferes with ATP production, resulting in the inhibition of endothelial cell growth (Moser et al., 1999). Finally, several data suggest that different domains may contribute to the overall anti-angiogenic function of angiostatin by their distinct anti-migratory and anti-proliferative activities (Cao et al., 1996).

Endostatin, a carboxy-terminal fragment of collagen XVIII, derived through elastase-mediated cleavage (Wen et al., 1999), was isolated from the conditioned media of hemangioendothelioma (EOMA) cells (O'Reilly et al., 1997). Endostatin specifically suppresses endothelial cell proliferation *in vitro* and increases the apoptotic rate in tumours 7-fold without affecting the proliferation rate of the tumour cells. *In vivo*, endostatin showed potent inhibitory activity against EOMA, Lewis lung, T241 fibrosarcoma, and B16F10 tumour cell lines. Interestingly, endostatin does not seem to induce drug resistance. Moreover, repeated cycles of systemic endostatin administration in tumour-bearing mice caused sustained tumour dormancy in the absence of further treatment (Boehm et al., 1997). Its anti-tumour activity is now being evaluated in phase I trials for a variety of solid tumours.

2.2.8 Tumour Vasculature: Morphology and characteristics

Tumour vasculature is functionally and morphologically different from tht vasculature in normal tissue. (McDonald and Baluk, 2002). Their global organization is abnormal. Tumour blood vessels are irregular, heterogeneous in distribution, larger in size and almost all of them are leaky. The capillaries are characterized by an irregular diameter, and are often dilated. The endothelial cells often overlap with each other and they are organized in a chaotic way. In addition they present an abnormal branching pattern, which leads to abluminal sprouts. Almost all of them lack a normal smooth muscle coat because the pericytes are absent or detached. There are very weak interconnections and focal intercellular openings between the endothelial cells. Scanning Electron Microscopy provided information that the openings are less than 2 μm in diameter. This is why the blood vessels are extremely leaky compared to normal vasculature, which is another important characteristic of tumour vessels. This leakiness can result on one hand in extravasation of plasma proteins and even erythrocytes, and on the other hand may lead to intravasation of tumour cells into the blood stream and the formation of metastases (Hendrix et al., 1999). As already mentioned above, the endothelial cells do not form a normal monolayer that would function as a normal barrier. That is why the blood vessels show increased endothelial permeability for small molecules but also for large ones. As a consequence of this drug penetration is very heterogeneous (Jain, 2001). The

tumour vessels have an irregular basement membrane regarding the matrix protein composition, assembly and structures. In addition, there is no clear division between arterioles and venules among tumour vessels. Blood flow is chaotic leading to a poorly oxygenated tumour tissue (Cao, 2004). Four regions are categorised on the basis of tumour vasculature: avascular necrotic regions with no vasculature; seminecrotic regions characterised by capillaries, precapilarries and post capillaries extended, without branching Toward the avascular necrotic region; stabilised microcirculation regions characterised by many venular and venous drainage vessels and few arteriolar vessels; and tumour advance front regions where flow is similar to percolation in porous medium (Endrich et al., 1979). Generally, the peripheral regions of a tumour show higher blood vessel density than the central regions. The ratio of avascular and seminecrotic regions to well-perfused region is also a function of tumour size. Larger avascular regions are present in larger tumours. The blood flow within a tumour is heterogeneous; blood flow is lower in the centre but higher in the periphery of the tumour relative to the blood flow in the surrounding normal tissues. All these features that characterize the tumour blood vessels are considered as very important key-points for Anti-angiogenic cancer therapeutics, anticancer drug delivery and targeting approaches.

2.2.9 Enhanced Permeation and Retention effect (EPR):

Tumour blood vessels exhibit marked extravasation and vessel wall hyperpermeability. Molecular transport across tumour vessels may occur via a number of pathways including interendothelial junctions and transendothelial channels, vesicular vacuole organelle, and fenestrations. The pore cutoff size of the tumour vasculature varies depending upon tumour model, however it is most commonly reported to range between 400 and 800nm (Yuan, 1995).

A critical advantage in treating cancer is the inherent leaky vasculature present serving cancerous tissues. The defective vascular architecture, created due to the rapid vascularization necessary to serve fast-growing cancers, coupled with poor lymphatic drainage allows an enhanced permeation and retention effect (EPR effect) (Teicher, 2000). The ability to target treatment to very specific cancer cells also uses a cancer's own structure in that many cancers overexpress particular antigens, even on their surface. This makes them ideal targets for drug delivery as long as the targets for a particular cancer cell type can be identified with confidence and are not expressed in significant quantities anywhere else in the body. (Brannon-Peppas and Blanchette, 2004)

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2.3 ANTIANGIOGENIC THERAPY

2.3.1 Antiangiogenesis : History

The concept of treating cancer by interfering with tumor vasculature is not new. In fact, as early as the nineteenth century, physicians and scientists were exploring this method of treatment. Walsh reported in 1844 that tumors could be cured if their circulation was artificially disrupted. Later, Goldman in 1907 described the chaotic growth of tumor vasculature, and discussed the critical role played by tumor vasculature and the possible therapeutic effects of its destruction. (Wootton, 2006)

The term of antiangiogenesis was first coined in early 1970s by Judah Folkman. He proposed the hypothesis that tumor growth would be halted if it were deprived of a blood supply as the expansion of the tumor mass beyond a size of a few cubic millimetres totally depends on neovascularisation that provides the growing tumor with oxygen and essential nutrients. This thesis, now supported by large direct or indirect experimental evidence, implies that tumors can potentially be starved to death by inhibiting their neovascularization. (Folkman, 1971; Keshet and Ben, 1999) Though the possible existence of angiogenesis inhibitors was first suggested in 1970s but it was not almost 10 years later than the first antiangiogenic molecules were identified. Protamine and platelet factor 4 were among the first angiogenesis inhibitors to be studied in early 1980s, followed by angiostatic steroids and then the discovery in 1985 that fumagillin and its synthetic analogues were angiogenesis inhibitors and subsequently that interferon α-2a was an AI (Folkman, 1997).

Anti-angiogenic therapies have become one of the most promising approaches in the anti-cancer drug development. Nowadays, several anti-angiogenic agents, alone or in combination with other conventional therapies, are in clinical trials based on the promising and successful preclinical research data (Jain et al, 2004).

Bevacizumab (Avastin®), a humanized variant of an anti-VEGF neutralizing monoclonal antibody, is the first antiangiogenic agent to be approved by the FDA in 2004 for the treatment of cancer (Ferrara et al., 2004; Kim et al., 1993). Bevacizumab was approved for the treatment of metastatic colorectal cancer (Hurwitz et al., 2004) and non-small cell lung cancer (Sandler et al., 2006) in combination with chemotherapy.

2.3.2 Antiangiogenic Therapy:

Antiangiogenic therapy represents a novel, potentially effective, and non toxic treatment for cancer. It is likely that these drugs will provide the next major breakthrough in the management of people with cancer. Antiangiogenic therapy will likely become part of the conventional treatment of cancer and will be used in combination with surgery, radiation therapy and chemotherapy.

Drug inhibition of angiogenesis is an area of intense research and at least 10000 cancer patients worldwide have received some form of experimental antiangiogenic therapy. More than 300 angiogenesis inhibitors have been discovered to date; 80 antiangiogenic drugs are currently in clinical trials, 12 of which target the key angiogenic factor vascular endothelial growth factor. A convincing regression of tumors has been reported for drugs against this target. Antiangiogenic therapy has raised the hopes both of cancer sufferers and of the physicians looking after them. Many of these agents may become available to people in the near future. (Srinivasan and Harris, 2002)

Antiangioenic therapy offers a number of advantages over traditional therapies for cancer :

- Tumor cells often mutate and become resistant to chemotherapy. Because antiangiogenic drugs only target normal endothelial cells, these cells are less likely to develop acquired drug resistance.
- All tumors rely upon host vessels. Antiangiogenic agents are therefore effective against a broad range of cancers.
- Conventional chemotherapy and radiotherapy indiscriminately attacks all dividing cells in the body, leading to side effects such as diarrhoea, mouth ulcers, hair loss, and weakened immunity. Antiangiogenic drugs selectively target dividing blood vessels and cause fewer side effects.
- Antiangiogenic drugs are relatively nontoxicand work at levels well below the maximum tolerated dose, so may be given in lower doses over longer periods of time.
- Antiangiogenic treatment may takes weeks or even months to exhibit its full beneficial effect, butthis allows for continuous, chronic control of disease.
- Antiangiogenic drugs may also serve as a powerful supplement to traditional chemotherapy or radiation therapy. (http://www.angio.org.)

2.3.3 Antiangiogenesis strategies:

Antiangiogenesis trials have been based on different strategies, the most important of them being:

a) strategies that interfere with angiogenic ligands, their receptors or downstream signaling

b) strategies that upregulate or deliver endogenous inhibitors or

c) strategies that are based on the direct targeting of tumor vasculature. (Ellis et al, 2003)

a) Inhibitorsfor Pro-angiogenic factors:

Blocking the binding of pro-angiogenic factors such as VEGF, bFGF and PDGF to their receptors is one strategy for anti-angiogenic therapy. These inhibitors not only block binding of ligands, but they shut down successive signaling as well, such as that triggered by the phosphorylation of tyrosine-kinase receptors. The goal of this strategy is to inhibit angiogenesis. VEGF is the most extensively researched target for anti-angiogenic therapy. The expression of its receptors is almost exclusively on endothelial cells and its upregulation on tumor endothelium compared with the adjacent normal endothelium are the two characteristics that make VEGF a very attractive target for this anti-cancer therapy approach. A fractional list of anti-angiogenic agents tried by different companies is given in Table 2.2 (Ellis et al, 2003).

Table 2.2 Anti-angiogenic agents (Ellis et al, 2003)

Agent	Company	Mechanism of action
2C6	ImClone Systems	Antibody to VEGF-R2
ABT- 510	Abbott Lab's	TSP-1analog
ABX- IL8	Abgenix	Antibody to IL- 8
Angiostatin	EntreMed	Unknown
Angiozyme	Ribozyme	Ribozyme targeting VEGF- R1
Avastin	Genetech Biotech.	Antibody to VEGF
CEP- 7055	Cephalon	TKI to VEGF- R1.-R2.-R3
Combrestatin A- 4 prodrug	Oxigene	Tubulin- binding agent
CNTO 95	Centocor	Antibody to avβ3 and avβ5 integrin
EMD- 121974	Merck KGA	Integrin antagonist
Endostatin	EntreMed	Unknown
PTK 787/ZK222584	Novartis	TKI to VEGF- R2
SU11248	SUGEN	TKI to VEGF- R2 and PDGF- Rβ
Thalidomide	Celgene	Unknown
Vitaxin II	MedImmune	Antibody to avβ3 integrin
VEGF trap	Regeneron	Soluble receptor hybrid of VEGF
ZD6126	AstraZeneca	Tubulin binding agent
ZD6474	AstraZeneca	TKI to VEGF- R2. EGF- R

b) Endogenous Angiogenic inhibitors:

Another group of anti-angiogenic agents presented in Table 2.2 are agents which are proteolytic fragments of precursor proteins produced endogenously in vivo, such as angiostatin, endostatin and thrombospondin (TSP). The angiostatin is secreted by tumor cells. Its complete anti-angiogenic mechanism is not fully elucidated yet, but it is believed that it inhibits endothelial cell migration and induces their apoptosis (Oku and Shimizu, 2004). Endostatin has a similar function with angiostatin inhibiting both proliferation and migration of endothelial cells (Oku and Shimizu, 2004). Thrombospondin is a high-molecular weight extracellular matrix glycoprotein and it is secreted by a variety of cells. There are five subtypes of thrombospondin: (TSP -1,TSP -2, TSP-3, TSP-4 and TSP-5). The first two subtypes, TSP-1 and TSP-2 are negatively correlated with vessel count in tumors and their analogs are now in clinical trials (Ellis et al., 2003).

c) Vascular- Targeting:

In contrast to anti-angiogenic agents, that aim to inhibit new tumor vessel formation, vascular-targeting agents attack the existing neovasculature in tumors (Eichhorn et al., 2004). The goal of using these kinds of agents is to induce tumor endothelial cell apoptosis and in addition microvessel thrombosis. Antibodies specifically targeting antigens expressed on tumor endothelium serve as one example of vascular-targeting agents.

Another example of vascular-targeting agents is the group of tubulin-binding agents, which selectively act on tumor vasculature and destroy it by polymerizing the endothelial cell microtubules. As a consequence, the cytoskeleton of endothelial cells is disrupted, resulting in microvessel thrombosis and secondary cell death (Blakey et al., 2002). Two very important vascular- targeting molecules that are now in clinical trials are Combretastatin-A-4 prodrug (OxiGene) and ZD6126 (AstraZeneca), both respectively in Phase I and II (Thorpe, 2004).

2.3.4 Different classifications for Angiogenesis Inhibitors (AIs):

Depending on above mentioned strategies the AIs can be classified as direct, indirect, or mixed inhibitors.

Direct AIs: Direct AIs target the ECs involved in the malignant disease by inhibiting their ability to proliferate, migrate, or form new blood vessels. The action of direct AIs may be independent of the type of cancer cell with low probability of acquired resistance.

Indirect AIs: Indirect AIs interfere with production of angiogenic factors by malignant cells, stromal cells, and inflammatory cells or with extracellular processes. Resistance to indirect

AIs may be more likely than resistance to direct AIs because they target genetically unstable tumor cells (Folkman, 2002).

Mixed AIs: Mixed AIs, such as multitargeting kinase inhibitors, epidermal growth factor receptor (EGFR) inhibitors or neutralizing agents, protein kinase C inhibitors, and others, as well as cytotoxic anticancer agents, target both tumor ECs and malignant cells.

Folkman (Folkman, 2002) proposed a classification system based on the efficacy of AIs in preclinical tumor models:

First generation AIs, such as interferons, TNP-470, thalidomide, and matrix metalloproteinases inhibitors (MMPIs), only slow tumor growth.

Second-generation AIs, such as anti-VEGF and anti-integrin $\alpha_v\beta_3$ antibodies, frequently produce tumor regression.

Third-generation AIs, such as angiostatin, endostatin, and TSP-1, can be curative in experimental tumors (Longo et al., 2002).

AIs can also be classified by the mechanism of action: inhibitors of angiogenic factors secretion, inhibitors of EC intracellular signaling transduction, inhibitors of EC proliferation, inhibitors of MMPs, agents cytotoxic toward ECs, and inhibitors of mobilization of EPCs from bone marrow (Kerbel, 2001).

Several AIs are currently under study in Phase I, II, and III clinical trials (http://www.cancer.gov/clinicaltrials) for current trials.

2.3.5 Combination therapy:

It appears likely that cancer therapy will be in most cases combinatorial. Antiangiogenic agents therefore, need to be combined with cytotoxic chemotherapy, radiation therapy and/or targeted therapies. Certain preclinical studies have documented advantages of combining AIs with cytotoxic chemotherapeutic agents or radiation therapy. These combined regimens produced additive or synergistic antitumor activity (Ferrara and Kerbel, 2005). There is considerable debate regarding the mechanisms of potentiation with chemotherapy. In fact it seems counterintuitive that agents, which reduce tumor blood flow such as angiogenesis inhibitors may enhance the efficacy of cytotoxic agents. One hypothesis postulates that antiangiogenic agents may, in some circumstances, 'normalize' the tumor vasculature, resulting in improved delivery of chemotherapy into tumor cells (Jain, 2005). This concept has been dealt in brief under section 2.3.6

Potentiation of the therapeutic effects with combined regimens may be related to increased access into the tumor mass of cytotoxic drugs or to enhanced oxygen pressure, as a result of the enhanced permeability induced by AIs. The greater-than-additive therapeutic effects may result from indirect effects on tumor ECs in addition to direct effects on tumor cells (Gasparini et al, 2006). The multitargeted kinase inhibitor SU11248 blocks the activity of receptor tyrosine kinases located on both ECs and malignant cells (Laird and cherrington, 2003). There were no clear benefits of using combinations of different AIs, and AIs were more effective in combination with cytotoxic therapies when used as two-agent combinations rather than as single agents (Kakeji and Tiecher, 1997). Combinations of TNP-470 with various cytotoxic chemotherapeutic agents, such as paclitaxel and carboplatin in non–small-cell lung cancer (NSCLC) and breast cancer models, paclitaxel in NSCLC, cisplatin in liver metastasis of human pancreatic cancer, and fluorouracil in liver metastasis of colorectal cancer, produced additive or synergistic antitumor activity (Satoh et al., 1998; Shishido et al., 1998, Ogawa et al., 2000). Also, it has been proposed that administration of lowdose chemotherapy at close regular intervals (metronomic therapy) preferentially damages tumor vessels such that the combination with antiangiogenic agents (eg. VEGF blockers) amplifies the antivascular effects, leading to enhanced killing of tumor cells (Kerbel, 2006).

Other potential rationals of combining AIs with chemotherapy listed as below:

Rationale of Combining AIs with Chemotherapy

- Antiangiogenic and cytotoxic agents work independently on different cellular targets
- AIs reduce interstitial pressure and favour tissue diffusion of chemotherapy
- Block of angiogenesis may reduce intratumoral hypoxia Anti-VEGF compounds also increase vessel permeability and extravasation of cytotoxic agents
- Reduced secretion of soluble endothelial growth factors (block of autocrine stimulation)
- Reduced secretion of soluble tumor growth factors (block of paracrine stimulation) (Gasparini et al., 2005)

Antiangiogenic therapies may also be combined with radiotherapy to improve local tumor control and to reduce the risk of metastases. Combinations of indirect AIs with radiation therapy can block tumor growth by inhibiting VEGF secretion stimulated by hypoxia (Gorski et al. 1999). In animals bearing Lewis lung carcinoma, the seminal studies by Teicher et al (Kakeji and Teicher ,1997; Teicher,1996; Herbst et al.,1998) demonstrated that TNP-470,50 minocycline, suramin, and genistein, alone or in two-agent combinations with cytotoxic

agents and radiation therapy, enhanced the regression of primary subcutaneous tumors and reduced the number and size of lung metastases (Teicher et al., 1992; 1994; 1995; Inoue et al., 2003). During a course of radiotherapy, some tumors increase their angiogenic activity (Ansiaux et al., 2005). Combined-modality therapies with antiangiogenic agents induce a normal microvascular bed out of the disorganized tumor vessels. There is a critical time during the antiangiogenic treatment when pericytes are recruited, the vascular basement membrane adopts a thinner morphology, and tumor oxygenation temporarily increases. This is a favorable time to apply ionizing radiation since it is preferentially lethal to replicating and well-oxygenated cells. The combination of an antiangiogenic agent and radiation therapy is optimally effective if this window of opportunity is exploited, and concerns regarding the induction of metastases have not been confirmed (Koukourakis et al., 2001; Ergun et al., 2003; Ma et al., 2003)

2.3.6 Normalization of Tumor Vasculature:

Jain proposed an additional rationale for anti-angiogenic therapy: 'normalizing' tumor vasculature before its destruction to improve the delivery of drugs and oxygen and thus positively help different anti-cancer treatments (Jain, 2005).

As already stated, tumor angiogenesis leads to formation of tortuous, dilated and saccular blood vessels that are poorly organized and hyperpermeable. Because of their specific morphological features, the blood flow is heterogeneous and and not equally distributed in tumor areas. In addition, the microenvironment generated by a heterogeneous blood flow is characterized by hypoxia, interstitial hypertension and acidosis. Hypoxia in itself is responsible for the fact that tumor cell become resistant to radiation and some cytotoxic drugs. Moreover it induces genetic instability and selects for more malignant cells with increased metastatic potential (Jain, 2005). These pathophysiological characteristics of solid tumors compromise the delivery and effectiveness of conventional cytotoxic therapies.

If immature and inefficient blood vessels could be pruned by eliminating excess endothelial cells, the resulting vasculature would be more 'normal' and hence more conducive to the delivery of nutrients and therapeutics. This 'normalized' vasculature would still be more permeable and less 'mature' than its normal counterpart.

In normal conditions, the inhibitors and the stimulators of Angiogenesis are well balanced. However, in pathological conditions the equilibrium between stimulators and inhibitors expression is altered in favor of stimulators and it this imbalance persists for quite a long time (Jain, 2005). Attempting to restore the equilibrium and not try to tip the balance in favor of

inhibitors can result in a normalization of the tumor vasculature (Figure 2.5). In doing so, certainly there are a lot of risks involved that might be overlooked. By not trying to inhibit the angiogenesis stimulators expression, one might think that the attack of tumor vasculature may be weaker and furthermore tumor regression may be impaired. Obviously this is true. But on the other hand, based on studies until recently, anti- angiogenic agents do require a very high dose to fulfill their function (Jain, 2005). This high- dose exposure leads in a lot of cases to adverse effects in the vasculature of normal tissue in the tumor- surrounding area, but also in the cardiovascular, endocrine and nervous system (Jain, 2005). An example is the anti- angiogenic treatment with Avastin, which is allied with a high- risk of arterial thromboembolic events (Yang et al., 2003). Consequently exposure to increased doses would result to more adverse effects which can be lifethreatening.

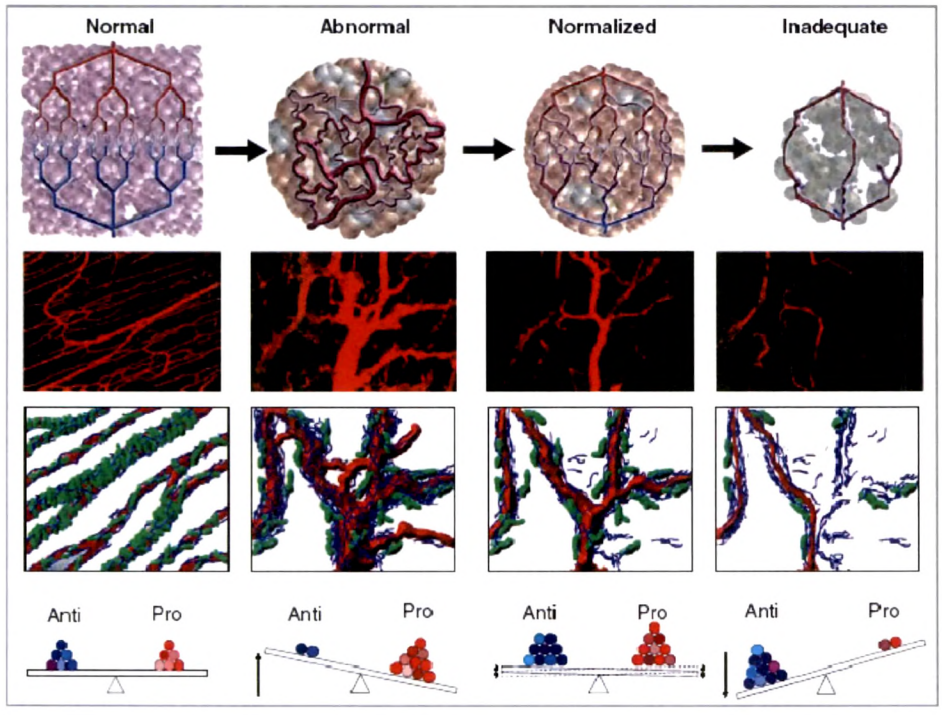

Figure 2.5: Tumor vessel normalization and tumor response. Illustration of transition from an abnormal vasculature to a normal one in tumors. The transition is accompanied with the re-established levels of the angiogenic factors (Adapted from Jain, 2005).

Moreover, by inhibiting the angiogenesis and cause vasculature regression, this will in turn influence drug and oxygen delivery to different parts of the tumor, and treatments using chemotherapy and radiation will not be efficient. In a nutshell, there is a need to very carefully find equilibrium between normalization and unnecessary vascular regression (Jain, 2005).

2.3.7 Criteria for Antiangiogenic Activity:

The degree of antiangiogenic activity is dose dependent. Most conventional chemotherapy drugs have some degree of antiangiogenic activity as a consequence of their cytotoxic activity. Ideal botanical derivatives would specifically antagonize new vessel formation in tumors, without significant toxicity to normal tissues and without major adverse reactions. The ideal agent would also inhibit tumor cell proliferation through other physiologic pathways, such as influencing intracellular signaling pathways. Multiple levels of antiangiogenic activity may be required to overcome the development of resistance by tumor-associated endothelial cells (TEC). Survival factors, such as the increased secretion of VEGF and bFGF by the tumor cells, activate intracellular pathways that prevent TEC apoptosis. Maximal antiangiogenic activity usually requires prolonged exposure to low concentrations of the active agent. This approach contrasts with the concept of administering maximum tolerated doses of cytotoxic drugs to maximize tumor cell kill. Some reports have confirmed the utility of combining low, frequent-dose chemotherapy plus an agent that specifically targets the endothelial cell compartment (Hanahan et al., 2000; Maraveyas et al., 2005). The evidence suggests that an antiangiogenic schedule can be more effective than using high-dose cytotoxic drugs alone. We hypothesize that concomitant scheduling of natural antiangiogenic with low, frequent-dose cytotoxic therapies may have biological advantages that can increase therapeutic gain.

2.3.8 Natural Antiangiogenic Agents:

Natural compounds are already being used in cancer treatment. For better or for worse, hundreds of thousands of patients around the world are experimenting with natural compounds in their efforts to heal themselves of cancer. There is solid evidence that these compounds inhibit cancer by interfering with one or more of the mechanisms that are central to cancer progression. Many natural polyphenolic compounds like (eg. Quercetin, Genestein, and Curcumin) have been shown to have antiangiogenic properties *in vitro* and *in vivo.* (Boik, 2001, Furness et al., 2005).

There were no clear benefits of using combinations of different AIs, and AIs were more effective in combination with cytotoxic therapies when used as two agent combinations rather than as single agents (Kakeji and Teicher, 1997). Single antiangiogenic agents seem to have limited efficacy. Natural health products contain a range of complex organic chemicals that may have synergistic activity. They may inhibit angiogenesis by interacting with multiple pathways, as well as having other activities that can interact with cell signaling, the apoptotic

pathway, and the interaction of cancer cells with the immune system. Some antiangiogenic agents also have anticoagulation activity that may also be associated with a reduction of metastases. Heparin is a well known example of a therapy with both anticoagulation and antiangiogenic activities. Instead of developing multiple monoclonal antibodies to target the various peptides and their receptors, an alternative approach would be to evaluate phytochemicals and some animal-derived chemical derivatives that influence multiple pathways. There is need for clinical research that evaluates their use as adjuvant therapy to conventional treatment with cytotoxic drugs and radiotherapy. Antiangiogenic natural health products may be most effective in impeding cancer recurrence after cytotoxic therapy, encouraging tumors to remain dormant by changing the balance from cell proliferation to cell death by apoptosis (Yance and Sagar, 2006).

Further research is necessary to screen herbs that may be useful antiangiogenic therapies. Table 2.3 lists natural health products with antiangiogenic activity (Singh and Agarwal, 2003).

Table 2.3 Natural Health Products With Potential Direct and Indirect Antiangiogenic Activity

Herbs and associated phytochemicals
Aloe barbadensis Mill. (Liliaceae) (aloe vera leaf and pulp extracts)
Angelica sinensis (aqueous extracts)
Artemisia ann-ua (artemisinin)
Camellia sinensis (epigallocatechin)
Chrysobalanus icaco L. (Chrysobalanaceae) (methanol extract)
Curcuma longa (curcumin)
Dysoxylum binectariferum Hook.f. ex Bedd (Meliaceae) (flavopiridol)
Flos magnoliaea (magnosalin)
Ganoderma lucidum (triterpenoids)
Ginkgo biloba (ginkgolide B)
Glycyrrhiza glabra L. (Fabaceae) (isoliquiritigenin; glabridin)
Hibiscus sabdariffa (protocatechuic acid)
Livistona chinensis R.Br. (Arecaceae) (aqueous extract from seed)
Matricaria chamomilla L. (Asteraceae) (flavonoids: apigenin, fisetin)
Ocimum sanctum (carnosol; ursolic acid)
Omega-3 fatty acids (eicosapentaenoic acid, docosahexaenoic acid)
Magnolia obovata Thunb. (Magnoliaceae) (honokiol)
Panax ginseng (saponins: 20(R)- and 20(S)-ginsenoside-Rg3)
Polypodium leucatomos Poir. (Polypodiaceae) (difur)
Poria cocos (1-3-alpha-D-glucan)
Polygonum cuspidatum Sieb. & Zucc. (Polgonaceae) (resveratrol)
Proanthocyanidin
Quercetin
Rabdosia rubescens (ponicidin and oridonin)
Rosmarinus officinalis (carnosol and ursolic acid)
Scutellaria baicalensis (baicalin and baicalein)
Silybum marianum (silymarin)
Soy isoflavones (genistein, daidzein)
Tanacetum parthenium Sch. Bip. (Asteraceae) (parthenolide)
Tabebuia avellanedae Lor. ex Gris. (Bignoniaceae) (□-lapachone)
Taxus brevifolia Nutt. (Taxaceae) (taxoids)
Viscum album (lectins)
Zingiber officinale (6-gingerol)
Other Chinese herbs

Thus keeping in mind the hypothesis behind the combination therapy of anticancer and antiangiogenic drug, in the present thesis, we have tried to explore the therapeutic benefit using Etoposide as cytotoxic drug and Quercetin Dihydrate as angiogenesis inhibitor by incorporating them separately into polymeric nanoparticles.

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2.4 NANOTECHNOLOGY IN CANCER

2.4.1 Introduction: Cancer Nanotechnology

Cancer nanotechnology is emerging as a new field of interdisciplinary research, cutting across the disciplines of biology, chemistry, engineering, and medicine, and is expected to lead to major advances in cancer detection, diagnosis, and treatment. The basic rationale is that metal, semiconductor, and polymeric particles have novel optical, electronic, magnetic, and structural properties that are often not available from individual molecules or bulk solids. Recent research has developed functional nanoparticles that are covalently linked to biological molecules such as peptides, proteins, nucleic acids, or small-molecule ligands. Medical applications have also appeared, such as the use of superparamagnetic iron oxide nanoparticles as a contrast agent for lymph node prostate cancer detection and the use of polymeric nanoparticles for targeted delivery to tumor vasculatures.

Figure 2.6 illustrates nanotechnology applications in cancer through molecular tumor imaging, early detection, molecular diagnosis, targeted therapy, and cancer bioinformatics (Nie et al., 2007).

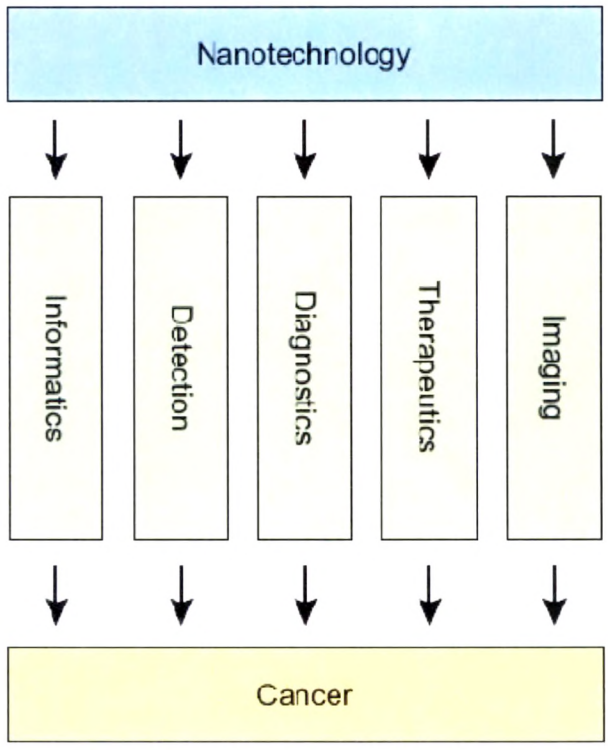


Figure 2.6 Schematic diagram showing nanotechnology applications in cancer.

Amongst the most promising delivery systems, the extensively researched colloidal delivery systems include liposomes, SLNs, polymeric micelles, dendrimers and nanoparticles as depicted in the Figure 2.7, nanoparticles are considered as promising colloidal drug carriers because of the versatility in terms of compounds that can be encapsulated and the degree to which these particles can be engineered and surface properties can be tailored. Nanoparticles also overcome the technological limitations and stability problems associated with other delivery systems. Thus this chapter focuses on the polymeric nanoparticles.

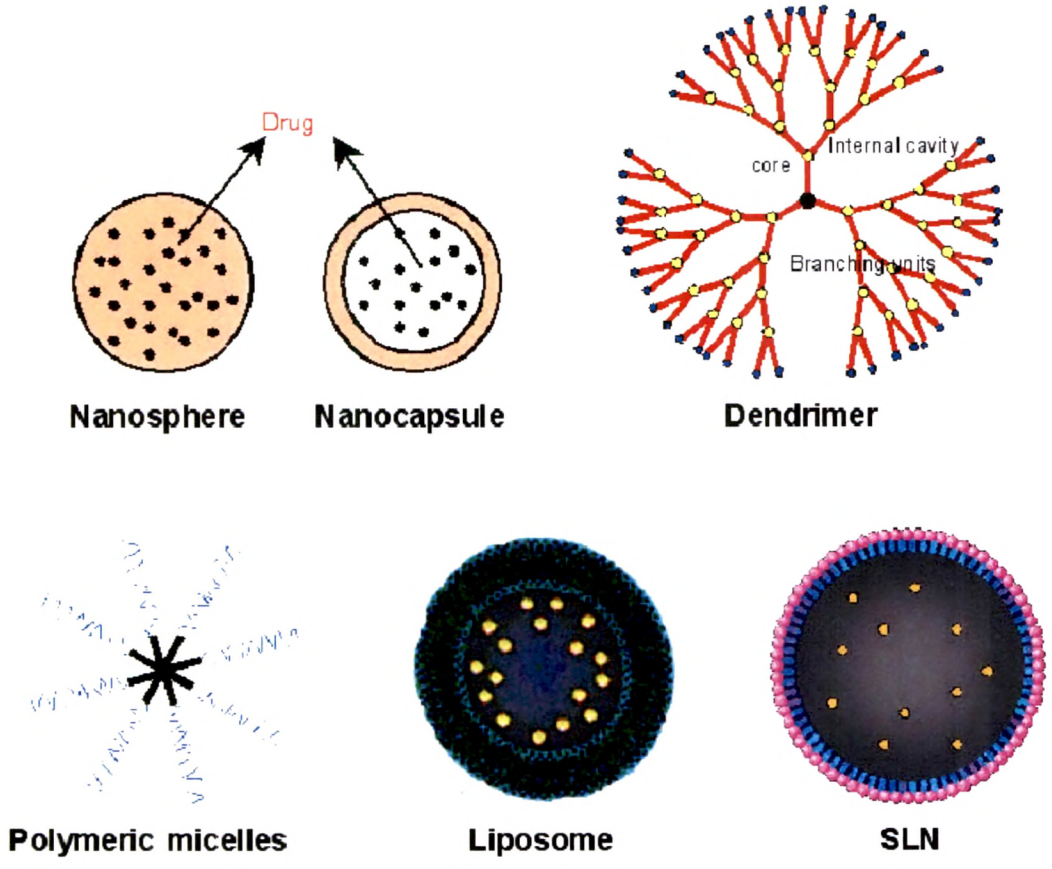


Figure2.7. Schematics of different nanotechnology based drug delivery systems for cancer therapy. (Adapted from Sahoo and Labhasetwar, 2003)

2.4.2 Polymeric Nanoparticles :

In January 2005, FDA approved ABRAXANE® for breast cancer treatment, the first nanoparticle system for drug delivery (http://www.abraxane.com, http://www.news-medical.net) This system, based on nanoparticle Albumin-bound (nab®) Paclitaxel, showed better and faster rate of shrinking tumors in 460 patients with metastatic breast cancer, almost

double compared with solvent-based Taxol®. It integrates biocompatible proteins with drugs to create the nanoparticle form of the drug having a size of about 100-200 nanometers to overcome insolubility problems encountered with paclitaxel. The solvent Cremophor- EL, used previously in formulations of paclitaxel, causes severe hypersensitivity reactions. With Abraxane, the active component (paclitaxel) can be delivered into the body at a 50% higher dose over 30 minutes. Because Abraxane is solvent-free, solvent-related toxicities are eliminated. In a randomized Phase III trial, the response rate of Abraxane was almost twice that of the solvent-containing drug Taxol (Pharmaceutical News, 2005).

Briefly, Nanoparticles may be defined as sub-nanosized colloidal structures composed of synthetic or semi-synthetic polymers that vary in size from 10 - 1000 nm. Depending upon the method of preparation, nanospheres (matrix systems) or nanocapsules (reservoir systems) can be obtained in which drug either is dissolved, entrapped, encapsulated or attached to the nanoparticle matrix. The drug can either be directly incorporated during polymerization or by adsorption onto preformed nanoparticles (Brigger and Couvereur, 2002).

Biopolymeric nanoparticles consist of a matrix of biopolymers that may be linked through intermolecular attractive forces or through chemical covalent bonds to form solid particles. Nanoparticles may consist of a single biopolymer or may have a core-shell structure (Figure 2.7). Because of the versatility in terms of compounds that can be encapsulated and the degree to which these particles can be engineered and surface properties can be tailored, they have rapidly become the most promising nanoscale delivery systems in the pharmaceutical and cosmetics industries.

2.4.3 Clearance mechanism and circulation time:

Following the systemic administration, the body normally distributes nutrients, clears wastes, and distributes drugs via the vascular and lymphatic systems. The systemic use of nano-capsule and nano-particles are limited by the presence of reticuloendothelial system (RES) which recognise them as foreign products and quickly removes them from blood circulation. Intravenously injected particles are cleared from the circulation by Kupffer cells and macrophages in a process that is triggered by deposition of blood opsonic factor and complement proteins on the surface of the injected particles (Moghimi and Szebeni, 2003).

Colloidal nano-particles are generally recognised by the macrophages due to the physicochemical properties, in particular size, surface charge and surface hydrophobicity. As compared to the hydrophilic nano-particles hydrophobic polymeric nano-particles are efficiently cleared from circulation (Claudia et al., 1998).

Various attempts have been made to achieve long blood circulation time. Recent studies showed that size and surface characteristics of injected particles influences clearance mechanism. Relative to the very small nano-particles, particles larger than 200 nm in diameter activate the complement system and cleared more rapidly (Moghimi and Szebeni, 2003). In principle clearance may be favourly modulated by the use of nano-particles with engineering characteristic tailored to the phenotype, physiological activity, and recognition of macrophages (Moghimi et al., 2005). For example coating the nanoparticles with hydrophilic polymer coatings such as PEG, poloxamines, poloxamers, polysaccharides, this avoids RES recognition by minimising the interaction with protein and opsonins. This prolongs the blood circulation time of nanoparticles and allows controlled release of therapeutics (Claudia et al.,1998). The circulation time of nano-particle can be enhanced by controlling its pharmacokinetic profile. The size of the particle must be large enough to avoid leakage into capillaries and not so large that they are susceptible to macrophages based clearance. The targeting mechanisms are briefly dealt below.

2.4.4 Mechanisms of targeting: Passive and Active

Passive targeting

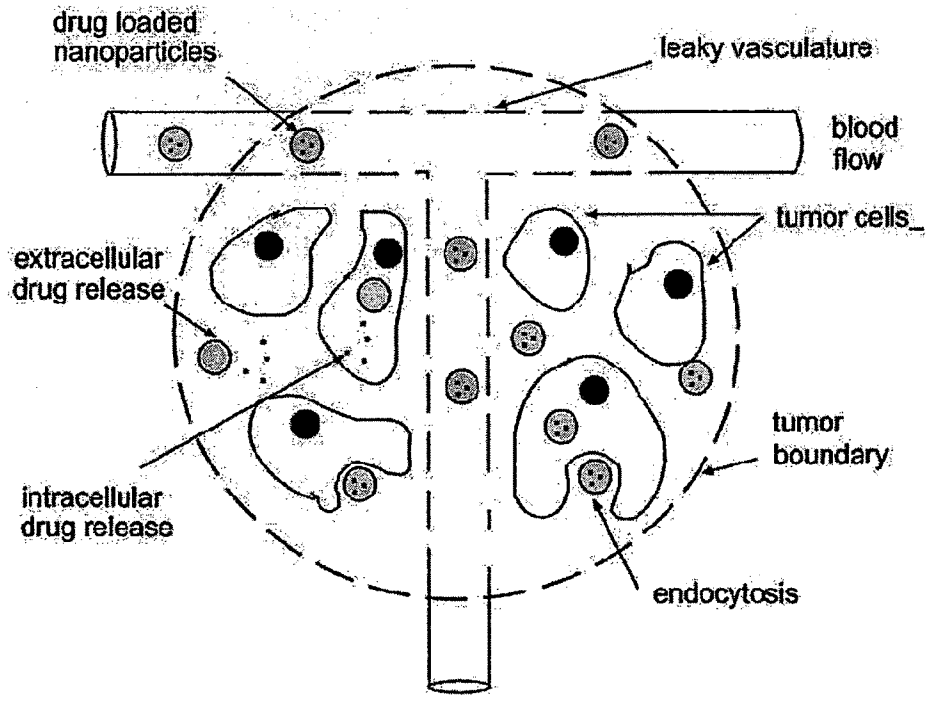


Figure 2.8 Pasive targeting, the EPR effect. Tumor tissues are known to have leaky vasculature and results in a passive accumulation of nanoparticles and this phenomenon is referred to as EPR.

Many times differences in the structure and behaviour of normal and tumor tissue helps in accumulation of nano-particles in solid tumors at much higher concentration than in normal tissue due to the enhanced permeability and retention (EPR) effect (Figure 2.8) (Ulbrich and Vladimir, 2004; Jain , 2001). In tumor, because of enhanced permeability of capillaries and limited lymphatic clearance result in accumulation of nano-particles. This may be one of the basic ideas behind designing of nano-capsule faciliting tumor specific delivery of drug. For such a passive targeting mechanism to work, the size and surface properties of drug delivery nanoparticles must be controlled to avoid uptake by the reticuloendothelial system (RES) (Gref et al., 1994). To maximize circulation times and targeting ability, the optimal size should be less than 100 nm in diameter and the surface should be hydrophilic to circumvent clearance by macrophages. Figure 2.9 explains how structure of tumor avoids clearance mechanism and improves retention time of nanoparticle in it. (Praetorius and Mandal, 2007; Ulbrich and Vladimir, 2004)

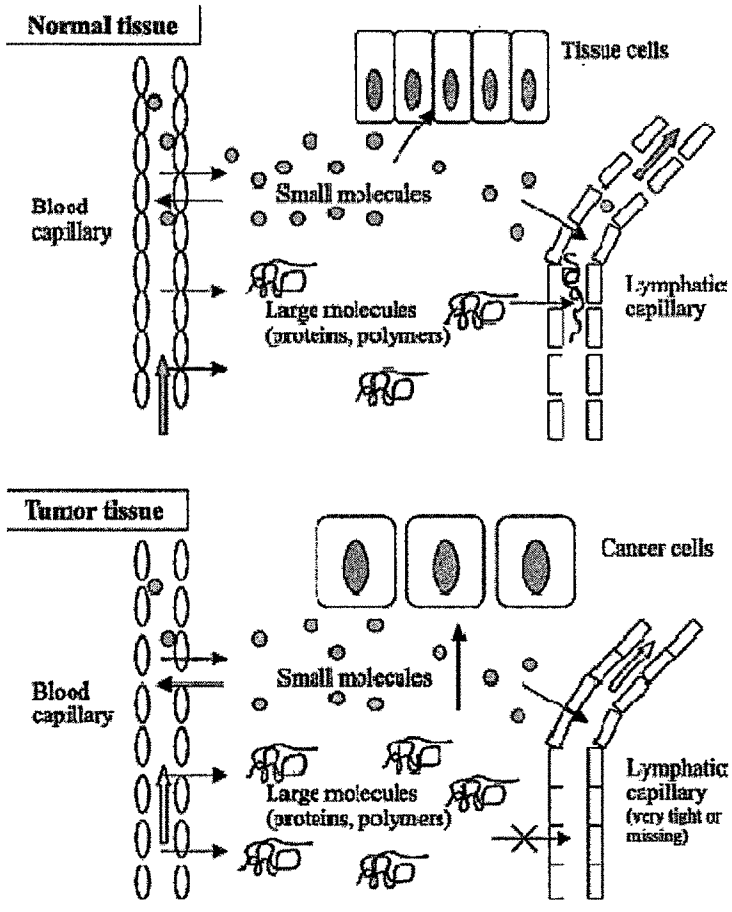


Figure 2.9: Clearance and retention of nanoparticles in normal and tumor tissue (Ulbrich and Vladimir, 2004)

Another passive targeting method is the direct local delivery of anticancer agents to tumors. This approach has the obvious advantage of excluding the drug from the systemic circulation. However, administration can be highly invasive, as it involves injections or surgical procedures. For some tumors, such as lung cancers, that are difficult to access, the technique is nearly impossible to use. (Nie et al., 2007)

Active targeting:

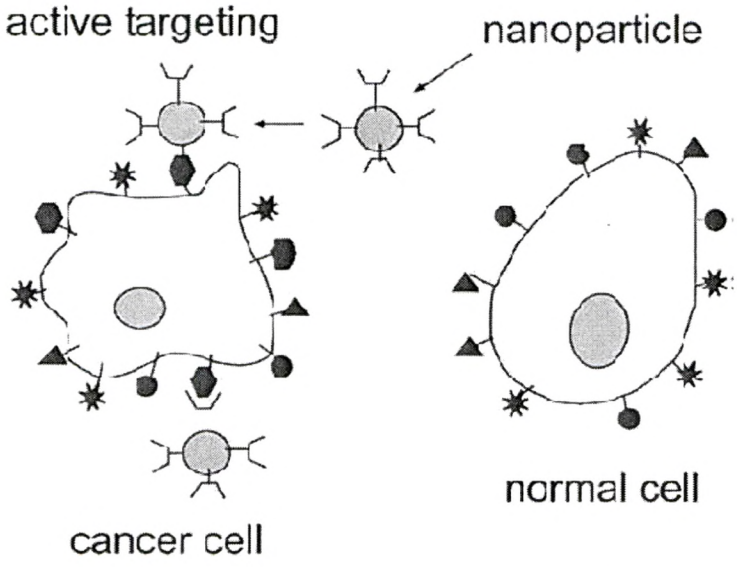


Figure 2.10: Active targeting. Nanoparticles with ligands or molecules attached to their surface can target tumor cells preferentially over healthy cells.

Active targeting has been performed to obtain a high degree of selectivity to specific tissues and to enhance the uptake of nanoparticles into target areas such as cancer cells and angiogenic microcapillaries growing around malignant cells (Figure 2.10). Nanoparticles are modified to target inherent characteristics of cancer cells such as rapid proliferation and particular antigen presentation (Moghimi et al., 2001). Nanoparticulate delivery systems utilizing specific targeting agents for cancer cells minimize the uptake of the anticancer agent by normal cells and enhance the entry and retention of the agent in tumor cells. Active targeting is usually achieved by conjugating to the nanoparticle a targeting component that provides preferential accumulation of nanoparticles in the tumor bearing organ, in the tumor itself, individual cancer cells, or intracellular organelles inside cancer cells. Thus these delivery systems include the anticancer agent, a targeting moiety-penetration enhancer, and a carrier. The types of molecules which are capable of specifically recognizing and binding to other biomolecules are receptors, receptor ligands, enzymes, and antibodies. In all cancer

therapies, targeting through surface modification provides numerous avenues for increasing treatment specificity and accuracy while reducing toxicity to healthy cells. (Praetorius and Mandal, 2007)

The overexpression of receptors or antigens in many human cancers lends itself to efficient drug uptake via receptor-mediated endocytosis. The cell surface receptor for folate is inaccessible from the circulation to healthy cells owing to its location on the apical membrane of polarized epithelia, but it is overexpressed on the surface of various cancers, including ovary, brain, kidney, breast, and lung malignancies (Leamon and Low, 2001; Leamon and Reddy, 2004). Folate receptors are often organized in clusters and bind preferably to the multivalent forms of the ligand. Furthermore, confocal microscopy demonstrated selective uptake and endocytosis of folate-conjugated nanoparticles by tumor cells bearing folate receptors.

Targeting of nano-capsule through angiogenesis has been the subject of intense research due to its role in cancer development. There are so many different molecules involved in angiogenesis which can be used as potential target for nano- capsule based delivery. Targeting the tumor vasculature is a approach that can allow targeted delivery to a wide range of tumors. In 2003 the first time clinical trial showed positive result for vascular targeting in patients with metastatic colorectal cancer. In this trial the antiangiogenic drug Avastin® targeted vascular endothelial growth factor (VEGF). Recent work in gene therapy has also worked to utilise VEGF as a target and liposome as a carrier (Brannon-peppas et al 2004). Recent work has also shown that gene delivery may also

be targeted to neovasculature by coupling lipid based cationic nano particles to an integrin alpha v beta –targeting ligand in tumor bearing mice (Brannon-peppas and Jmaes, 2004).

2.4.5 Polymeric Nanoparticles :

A basic requirement for the use of nanoparticles as drug delivery systems for human therapy is their biodegradability and biocompatibility. Another challenge for the use of nanoparticles as drug delivery systems is to minimize their side effects in the biological system in which dispersed. A controlled size distribution (monodisperse distribution of size), for accurate drug administration, is a central need for the use of nanoparticles in drug delivery systems. Moreover, the absence of toxic residues in the final nanosystem is required, and therefore stronger restrictions to the type of methods used for nanoparticles formation exist. Additionally, the stability of the nanoparticles should be addressed if parenteral administration of the nanoparticle is used. The aggregation process due to dispersion forces (i.e. electrostatic, hydrogen bonding, hydrophilic/hydrophobic, steric Van-der-Waals) is the

principal drawback of nanoparticle use in drug delivery. Therefore, the understanding of the complexity of the nanosystem, the biological system, and the interactions between the two is a basic requirement for successful implementation of new nano-systems designed for drug delivery.

2.4.5.1Materials

Polymeric materials exhibit several desirable properties including biocompatibility, biodegradability, surface modification, and ease of functionalization of polymers. Polymeric systems allow for a greater control of pharmacokinetic behaviour of the loaded drug,leading to more appropriate steady levels of drugs (Peppas, 1995).

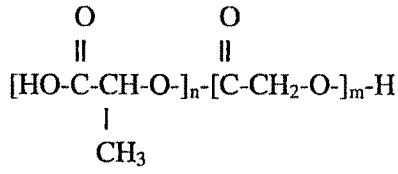
Polylactic acid, a key component of many biodegradable nanoparticles, was first developed in 1932, but its high cost and susceptibility to hydrolytic breakdown were believed to make it unsuitable for use in biomedical or agricultural applications or sparingly used in research (Lunt, 1998). However, the use of this polymer as an ideal material for sutures was discovered in the 1970s, and a process was developed in the 1980s to produce the polymer via bacterial fermentation, greatly reducing costs and increasing production rates.

A wide spectrum of synthetic and natural polymers is available for nanoparticle formation, but their biocompatibility and biodegradability are the major limiting factors for their use in the drug delivery area. Natural polymers are more restricted due to variation in their purity. Also, some natural polymers require crosslinking, which can inactivate the entrapped drug (Hans and Lowman, 2002). Synthetic polymers, on the other hand, offer better reproducibility of the chemical characteristics of the synthesized nanoparticles as compared to the natural polymers. Among these are chitosan, a natural antimicrobial and antioxidative polymer obtained from crustacean shells (Shahidi and Abuzaytoun, 2005) and the synthetic polymers from the ester family, such as poly(lactic acid), poly(-hydroxybutyrate), poly(caprolactone), poly(dioxanone), or other families such as poly(cyanoacrylates), poly(acrylic acid), poly(anhydrides), poly(amides), poly(ortho esters), poly(ethylene glycol), and poly(vinyl alcohol) are suitable for drug delivery due to their biodegradability, special release profiles and biocompatibility (Ghosh, 2004; Rawat, 2006).

2.4.5.2 Polymer PLGA: Poly(lactide-co-glycolide acid)

Various polymers have been explored as sustained release and protective carrier of drugs to a target site and thus increase the therapeutic benefit, while minimizing the side effects. Among these polymers, the thermoplastic aliphatic poly esters like PLA, PGA and especially PLGA

have generated tremendous interest due to their excellent biocompatibility and biodegradability.



PLGA and PLA have been approved by the FDA for numerous clinical applications such as major components in biodegradable sutures, bone fixation nails and screws, abdominal mesh and extended-release pharmaceuticals (Gombotz and Pettit,1995; Moghimi et al., 2001).

Many biodegradable systems rely on the random copolymers of PLGA, or the homopolymer PLA. These classes of polymers are highly biocompatible and possess good mechanical properties for drug delivery applications. It is a well-characterized polymer, which degrade chemically by hydrolytic cleavage of the ester bonds in the polymer backbone. Its degradation subproducts, lactic acid and glycolic acid, are water soluble, non toxic products of normal metabolism that are either excreted or further metabolised to carbon dioxide and water in the Krebs cycle (Okada et al., 1989).

PLGA and PLA belong to a broad class of polymers known as polyesters, and can be synthesized by a polycondensation reaction, or ring opening polymerization. Ring opening polymerization is currently the preferred method for synthesis for PLGA and PLA due to shorter reaction times and higher monomer conversion rates.

It provides controlled drug release profiles by changing the PLGA copolymer ratio which affects the crystallinity (low crystallinity, more amorphous polymer means more fast degradation) of PLGA (Gombotz and Pettit, 1995; Anderson and Shive, 1997; Moghimi et al.,2001; Ghosh, 2004; Bala et al., 2004). Glycolic acid monomeric units, greatly influences the degradation rate. A higher proportion of glycolic acid monomers incorporated into the copolymer will increase the degradation rate, and subsequent erosion, by increasing the hydrophilicity of the polymer and allow more biological fluids to penetrate the polymer matrix. The different ratios of lactide and glycolide exhibit different degradation rates, and thus can be tailor made for specific applications requiring specific degradation kinetics ranging from weeks to months.

For these reasons, PLGA has been selected as the polymer of choice in the present research. PLGA of different molecular weights (from 10 kDa to over 100 kDa) and different copolymer molar ratios (50:50, 75:25, and 85:15) is available on the market. Molecular weight and copolymer molar ratio influence the degradation process and release profile of the

drug entrapped. In general, low molecular weight PLGA with higher amounts of glycolic acid offer faster degradations rates (Anderson and Shive, 1997; Alexis, 2005)

2.4.6 Preparation methods:

First polymer nanoparticles for pharmaceutical applications were prepared in the late 1960.s and early 1970.s and were based on acrylamide micelle polymerisation. Since then, various polymerization methods as well as methods involving the use of preformed polymers have been developed and studied (Kreuter, 1994).

Methods available for PLGA nanoparticle synthesis can be divided into two classes, namely those based on polymerisation (bottom-up) and those taking advantage of preformed polymers (top-down techniques).

The bottom-up techniques such as emulsion or microemulsion polymerization, interfacial polymerization, and precipitation polymerization, employ a monomer as a starting point. In polymerisation methods, the monomers are polymerised to form the encapsulating polymer of the nanospheres or nanocapsules during the process (Allémann et al., 1993). Small particle sizes, ranging from 50 to 300 nanometers have been achieved by emulsion polymerisation (Kreuter, 1983; Schroeder et al., 1998). Drug amount in the nanoparticles has been reported to vary from less than 1% (w/w) to more than 10% (w/w), depending on the solubility of the drug (Allémann et al., 1993; Beck et al., 1994).

2.4.6.1 Polymerization method:

In this method, monomers are polymerized to form nanoparticles in an aqueous solution. Drug is incorporated either by being dissolved in the polymerization medium or by adsorption onto the nanoparticles after polymerization completed. The nanoparticle suspension is then purified to remove various stabilizers and surfactants employed for polymerization by ultracentrifugation and re-suspending the particles in an isotonic surfactant-free medium. This technique has been reported for making polybutylcyanoacrylate or poly (alkylcyanoacrylate) nanoparticles (Zhang et al., 2001; Boudad et al., 2001). Nanocapsule formation and their particle size depends on the concentration of the surfactants and stabilizers used (Puglisi et al., 1995).

Drawbacks which have limited the use of polymerisation methods for the synthesis of drug nanoparticles include the need to use surfactants to stabilise the emulsion during polymerisation, toxic or reactive residues of organic solvents, residues of initiators or unreacted monomers, risk of a chemical reaction between the drug molecule and the reactive

monomer, and the formation of oligomers. The residues from the synthesis require extensive purification work to result in a pharmaceutically acceptable product (Allémann et al., 1993; Couvreur and Puisieux, 1995). By using preformed polymers for the preparation of nanoparticles many of the problems involved in the polymerisation methods can be avoided (Allémann et al., 1993). Thus top down methods for preparation of PLGA nanoparticles are discussed below briefly.

2.4.6.2 Preformed polymer dispersion methods:

Top-down techniques are emulsion evaporation, emulsion diffusion, solvent displacement, and salting out, in which the nanoparticles are synthesized from the pre-formed polymer.

Probably the most common method to manufacture drug-containing polymer particles from preformed materials involves first dissolving the drug and the polymer into a water-immiscible solvent, such as dichloromethane or chloroform and forming a submicronic oil-in-water emulsion, for example by sonication. The organic solvent is evaporated using elevated temperature or reduced pressure (Allémann et al., 1993; Couvreur and Puisieux,1995; Hans and Lowman 2002, Bodmeier and Chen,1990; 1991). The resulting particle size and size distribution are determined by the emulsion droplet size and size distribution. Generally, particle sizes from 100 to 800 nanometers have been reported (Bodmeier and Chen, 1990; Krause et al., 1985)

Reported drug loadings vary up to 50% (w/w) by emulsion method (Bodmeier R 1990, Krause et al., 1985) and up to 15% (w/w) by solvent displacement method (Gamisans et al. 1999).

a) Emulsion Diffusion Method:

In this synthetic scheme, the polymer (PLGA) is dissolved in an organic phase (e.g., benzyl alcohol, propylene carbonate, ethyl acetate), which must be partially miscible in water. The organic phase is emulsified with an aqueous solution of a suitable surfactant (i.e. anionic sodium dodecyl sulfate (SDS), non-ionic polyvinyl alcohol (PVA), or cationic didodecyl dimethyl ammonium bromide (DMAB), under stirring. The diffusion of the organic solvent and the counter diffusion of water into the emulsion droplets induce polymer nanoparticle formation (Quintanar et al., 1998).

Important parameters that affect the nanoparticle size synthesized by emulsion evaporation are: PLGA copolymer ratio, polymer concentration, solvent nature, surfactant polymer molecular weight, viscosity, phase ratios, stirring rate, solvent nature, temperature and flow of water added.

b) Salting Out Method:

In this synthesis method, the polymer is dissolved in the organic phase, which should be water-miscible, like acetone or tetrahydrofuran (THF). The organic phase is emulsified in an aqueous phase, under strong mechanical shear stress. The aqueous phase contains the emulsifier and a high concentration of salts which are not soluble in the organic phase. Typically, the salts used are 60% w/w of magnesium chloride hexahydrate (Konan et al., 2002; Zweers et al., 2004) or magnesium acetate tetrahydrate in a ratio of 1:3 polymer to salt (Eley et al., 2004). Contrary to the emulsion diffusion method, there is no diffusion of the solvent due to the presence of salts. The fast addition of pure water, to the o/w emulsion, under mild stirring, reduces the ionic strength and leads to the migration of the water-soluble organic solvent to the aqueous phase inducing nanosphere formation (Barrat et al., 2000). The final step is purification by cross flow filtration or centrifugation to remove the salting out agent. Common salting out agents are electrolytes (sodium chloride, magnesium acetate, or magnesium chloride) or non-electrolytes, such as sucrose (Avgoustakis, 2004).

Important parameters to be considered are: polymer concentration and molecular weight, stirring rate and time, nature and concentration of surfactant and solvent, and cryoprotectans.

c) Emulsion Evaporation Method:

Emulsion evaporation is the oldest method used to form polymeric nanoparticles from preformed polymers. The method is based on the emulsification of an organic solution of the polymer in an aqueous phase followed by the evaporation of the organic solvent. The polymer is dissolved in a suitable solvent (e.g., ethyl acetate, chloroform, methylene chloride). The organic phase or aqueous phase is poured into the continuous phase (aqueous or organic phase) in which a surfactant is dissolved to impart stability to the emulsion. Emulsification is carried out under high-shear stress to reduce the size of the emulsion droplet (directly related with the final size of the nanoparticles). The process of emulsification is followed by evaporation of the organic solvent under vacuum, which leads to polymer precipitation and nanoparticle formation.

Normal emulsions oil in water (o/w) or water in oil (w/o) and double emulsions (w/o/w) can be used to accommodate the entrapment of active components with different properties. The o/w emulsion is used for entrapment of hydrophobic compounds, whereas w/o/w double emulsion is used for the entrapment of hydrophilic compounds. The method is widely used for microencapsulation because it is easy to scale up, it doesn't require high shear stress, and it can be adjusted (by use of the double emulsion method) to encapsulate water soluble drugs

(Alex and Bodmeier, 1990; Obeidat and Price, 2003; Mohammed and Hassan, 2003; Takada et al., 2003; Park and Kim, 2004).

d) Nanoprecipitation (Solvent Diffusion, or Solvent Displacement) Method:

Typically, this method is used for hydrophobic drug entrapment, but it has been adapted for hydrophilic drugs as well. Polymer and drug are dissolved in a polar, watermiscible solvent such as acetone, acetonitrile, ethanol, or methanol. The solution is then poured in a controlled manner (i.e. drop-wise addition) into an aqueous solution with surfactant. Nanoparticles are formed instantaneously by rapid solvent diffusion. Finally,the solvent is removed under reduced pressure (Mohanraj and Chen, 2006).

Important parameters to be considered are: polymer/surfactant ratio, polymer concentration, surfactant nature and concentration, solvent nature, viscosity, additives, active component, and phase injection (Bilati et al., 2005).

• Polymer concentration

The polymer concentration is maintained in the range of 1% w/v up to 10% w/v. Prakobvaitayakit and Nimmannit (2003) tested three different concentrations of 50:50 PLGA for nanosphere formation. The nanoparticle mean size was 190 nm for 1 % w/v, and the size increased to 238.9 nm for 10% w/v. Govender et al. (1999) used a concentration of 1% w/v to synthesize nanospheres of 157.1 ± 1.9 nm in size. Csaba et al. (2005) worked with a polymer concentration of 5 % w/v to form nanoparticles with a size range from 161 ± 7 nm to 269 ± 11 nm. This size range is correlated to the presence of different polymers in the polymer blend used (detailed in the surfactant section). Niwa et al. (Niwa et al., 1993) used 0.77% w/v PLGA forming nanospheres with a mean size of 224 ± 14 nm. Ameller et al. (Ameller et al., 2004) worked with a concentration of 2% w/v and obtained nanospheres with a mean size of 260 ± 50 nm, approximately (0.1 %w/w of poloxamer-188 was in the aqueous phase)

• Polymer molecular weight and copolymer ratio

The polymer molecular weight affects the size more significantly than the copolymer ratio, as follows. Niwa et al. (1993) worked with different molecular weights, and copolymer ratios. The PLGA 50:50 with a MW of 66475 Da formed nanospheres with a size of 338 ± 67 nm, which was similar to the nanospheres size of 85:15 PLGA with a MW of 66671 Da, measuring 385 ± 51 nm in size. The 85:15 PLGA with a MW of 127598 Da formed nanospheres with mean size of 637 ± 40 nm. These nanospheres were prepared with a mix of chloroform and acetone for the entrapment of Indomethacin.

• Solvent nature

The selection of good solvents to form smaller nanoparticles and to improve the entrapment efficiency of the active component is a complex and an important process. There is no clear definition of the 'best solvent' for this method. Niwa et al. (1993) used a mix of organic solvents (acetone, methanol, dichloromethane, or chloroform) to dissolve PLGA and drugs (indomethacin and 5-fluorouracil). The size of the 85:15 PLGA nanoparticles of two molecular weights (12279 Da and 66671 Da) changed when the mix of solvents was altered from 0.5:5:5 ml to 0.5:25:5 ml (dichloromethane /acetone /methanol). The first mix of solvents formed nanospheres with a mean size of 283 ± 37 nm and 213 ± 13 nm for the two molecular weights tested, and the second one formed nanospheres of 195 ± 34 nm, and 207 ± 13 nm. The reduction in size with increased acetone concentration is attributed to the reduction in the surface tension of the dichloromethane solution in the presence of acetone. The formation process performed with dichloromethane or chloroform formed nanospheres 1 μm and bigger in size. Acetone is commonly used alone for the preparation of nanospheres. Ameller et al. (2004, 2003) obtained a mean size nanoparticles of 258 ± 97 nm with zeta potential of -53.4 ± 0.5 mV. Prakobvaitayakit and Nimmannit (2003) formed nanospheres with a mean size varying from 190 nm to 643.9 nm. Panagi et al. (2001) formed nanospheres with mean size of 154 ± 23.5 nm, polydispersity of 0.489, and zeta potential of 45.1 ± 1.9 mV with the same solvent. Oster et al. (2004) obtained a mean size of 152 ± 3 nm and zeta potential of 35 ± 3 mV. Saxena et al. (2004) added methanol to acetonitrile (in which PLGA was dissolved) for a good dissolution of the active component. The mean size was 357 ± 0.21 nm with zeta potential of -16.3 ± 1.5 mV. The higher zeta potential (less negative) is attributed to the presence of PVA over the nanosphere surface. Csaba et al. (2004) worked with ethanol (organic phase) for the polymer nanoprecipitation. The mean size of the nanospheres (PLGA 50:50) obtained was 191.5 ± 7.1 nm. Other works used acetonitrile as the organic solvent. For example, Govender et al. (1999) prepared nanospheres with a size of 157.1 ± 1.9 nm with acetonitrile.

• Surfactant

A variety of surfactants are used for nanoparticle formation and stabilization. The surfactant can be anionic, cationic or nonionic. Surfactants in the poloxamer and poloxamines family, formed with polyoxiyethylene and polyoxypropylene, are commonly used in nanoparticle synthesis. Surfactants of different HLB values can be obtained by varying the amount of monomers; less ethylene oxide monomers and more propylene oxide monomers form surfactants with lower HLB values. Csaba et al. (2004) used poloxamer and poloxamines

blended with PLGA in the organic phase. The samples formed with more hydrophobic surfactants (HLB of 1 and 2.5) had an increased final size of up to 333.7 ± 82.1 nm for a mass ratio PLGA:surfactant of 50:75 mg/mg. The lower size nanoparticle formed was 159.8 ± 6.5 nm for the blend PLGA:Pluronic® F68 (HLB value of 29) of 50:75 mg/mg. Pluronic® F68 has shorter ethylene oxide chains and larger propylene oxide chains compared with the other surfactants tested. Ameller et al. (2003) used poloxamer 188 with a concentration of 0.1% w/w forming PLGA nanospheres of 262 ± 52 nm mean size. The zeta potential obtained was -11 mV. Another important surfactant used is PVA. Niwa et al. (1993) tested different concentrations of PVA. The range tested was from 0.5 % to 2 % of PVA in the aqueous suspension leading to nanoparticle formation with a mean size of 300 nm (not significant difference in the range tested). Saxena et al. (2004) obtained mean nanoparticle size of 357 ± 0.21 nm using 88 - 89 % hydrolyzed PVA.

• Active component entrapment

Entrapment of active components has an important effect on the final nanospheres final size; as a general rule, entrapment of hydrophobic active components leads to formation of smaller nanospheres, as compared to the entrapment of hydrophilic components. The interaction between solvent, polymer and active component must be taken into account to improve the drug loading and the drug entrapment efficiency.

The entrapment of procaine hydrochloride (with a pH of 5.8 for aqueous solution) was found to increase the nanoparticle size from 157.1 ± 1.9 nm to 209.5 ± 2.7 nm for a theoretical drug loading of 0% to 10%, respectively. The drug content increased from 0.2 to 4.6% w/w when the theoretical drug loading was increased from 1% to 10% w/w, but the entrapment efficiency decreased from 14.5% to 6.3% (Govender et al., 1999). Although, they reduced the nanosphere mean size by change of the aqueous pH (buffer at pH 9.3), the size for PLGA alone was 123.6 ± 2.3 nm, and for nanospheres with 10% w/w theoretical drug loading, the size was 186.5 ± 2.3 nm. In both cases, the entrapment formed bigger nanospheres in the presence of the drug, as compared with the PLGA alone. The nanospheres size was reduced with the entrapment of procaine dehydrate. When the theoretical drug loading of procaine dyhidrate was increased up to 10% w/w, the mean size was reduced from 157 ± 1.9 nm to 56.2 ± 1.9 nm. The drug entrapment efficiency ranged from 36.2% up to 44.1% (Govender et al., 1999).

• Phase injection

The organic phase addition to the continuous aqueous phase should be controlled and constant, by mild stirring, to assure a uniform distribution and diffusion. Prakobvaitayakit and Nimmannit (2003) used a constant flow rate of 0.3 ml/min with mechanical stirring of 750 RPM. In the Govender et al. (1999) work, they reported a drop wise organic phase addition. The stirring was done by a magnetic stirrer. The same procedure was followed by Saxena et al. (2004). Csaba et al. (2004, 2005) used vortex agitation for mixing both phases getting a fast organic phase dispersion and further moderate magnetic stirring. Other works using fast organic phase dispersion is that by Ameller et al. (2004, 2003).

Advantages

• The use of non highly toxic solvents (i.e. acetone).

• Reduced energy consumption because it only requires regular stirring. The process does not require high stress shear (i.e. sonication or microfluidization).

• Additives can be used for nanoparticle size reduction.

Disadvantages

• The solvent is removed by evaporation (time consuming).

• The main drawback is the requirement of drugs that are highly soluble in polar solvents (i.e. acetone, ethyl acetate), but they should be slightly soluble in water to minimize losses during solvent diffusion. The drug can diffuse to the aqueous phase reducing the drug entrapped in the PLGA nanospheres (De Jaeghere et al.,1999).

• The drug loading efficiency is lower for the hydrophilic drugs than hydrophobic ones because of their poor interaction (hydrophobic interaction) with the polymer leading to diffusion of the drug during the solvent displacement from the polymer in the organic phase to the external aqueous environment (Bala et al., 2004).

• Nanoparticle size is very much affected by the polymer concentration; higher nanoparticle sizes are obtained at higher polymer concentrations.

2.4.7 Characterisation of nanoparticles:

2.4.7.1 Morphology:

The methods most broadly used to characterize nanoparticle morphology are transmission electron microscopy (TEM), scanning electron microscopy (SEM), cryogenic transmission electro microscopy (cryo-TEM) and atomic force microscopy (AFM). TEM is used for shape, aggregation, and internal details. It is common to use a negative staining with phosphotungstic acid solution (3% w/v, adjusted to pH 4.7 with KOH) (Riley et al.,1999).

Panyam et al. (2003) used negative staining with uranyl acetate for TEM. SEM is used for surface characterization (shape, distribution, aggregation) with a layer of gold or nanoparticles alone (Quintanar et al., 1997). Cryo-TEM is used to observe the micellar formation of PLGA-g-PEG (Jeong et al., 2003). Ravi Kumar et al. (2004) and Saxena et al. (2004) used AFM for size and morphology of nanoparticles. .

2.4.7.2 Particle size and Distribution:

Particle size and size distribution are the most important characteristics of nanoparticle systems. They determine the *in vivo* distribution, biological fate, toxicity and the targeting ability of nanoparticle systems. In addition, they can also influence the drug loading, drug release and stability of nanoparticles.

Currently, the fastest and most routine method of determining particle size is by Photon-correlation spectroscopy (PCS) or dynamic light scattering and Laser Diffractometry (LD). The PCS method is useful for the detrmination of smaller particles, while the latter method is useful for the determination of larger particles. PCS requires the viscosity of the medium to be known and determines the hydrodynamic diameter of the particle by Brownian motion and light scattering properties (Swarbrick and Booylan, 2002). The measured size is influenced by the interaction of the particles with the surrounding liquid medium. The results obtained by photon-correlation spectroscopy are usually verified by SEM or TEM.

The electron microscopy methods also allow the exact particle determination. SEM requires the coating of dry sample with a conductive material such as gold. However the thickness of the gold coating is difficult to determine and hence may result in particle sizes more than the normal. TEM, with or without staining is a relatively easier method of particle size determination.

New types of high resolution microscopes such as atomic force microscope, force laser microscope and scanning tunnelling microscope are also useful in the investigations of nanoparticle surfaces.

Information about the nanoparticle structure and crystallinity may be obtained by X-ray diffraction and thermoanalytical methods such as differential scanning calorimetry, differential thermal analysis, thermogravimetryand thermal optical analysis (Gedde, 1990).

2.4.7.3 Surface property: Zeta potential

The zeta potential of a nanoparticle is commonly used to characterise the surface charge property of nanoparticles (Couvreur et al., 2002). It reflects the electrical potential of particles and is influenced by the composition of the particle and the medium in which it is dispersed. Nanoparticles with a zeta potential above (+/-) 30 mV have been shown to be stable in

suspension, as the surface charge prevents aggregation of the particles. The zeta potential can also be used to determine whether a charged active material is encapsulated within the centre of the nanocapsule or adsorbed onto the surface. Laser doppler anemometry is used to measure the zeta potential.

2.4.7.4 Drug loading and entrapment efficiency:

Ideally, a successful nanoparticulate system should have a high drug-loading capacity, thereby reduce the quantity of matrix materials for administration. Drug loading can be done by two methods:

- Incorporating at the time of nanoparticles production (incorporation method)
- Absorbing the drug after formation of nanoparticles by incubating the carrier with a concentrated drug solution (adsorption/absorption technique).

Drug loading and entrapment efficiency very much depend on the solid-state drug solubility in matrix material or polymer (solid dissolution or dispersion), which is related to the polymer composition, the molecular weight, the drug polymer interaction and the presence of end-functional groups (ester or carboxyl) (Govender et al., 1999; 2000, Panyam et al., 2004). The entrapment into the nanoparticles is described by two important parameters:

theoretical drug loading, which is the ratio between mass of drug used in synthesis and mass of polymer used in synthesis, and nanoparticle recovery, which is the ratio between mass of nanoparticles recovered and mass of polymer and drug used in synthesis. The drug content is calculated by the ratio of mass of drug in nanoparticles to mass of nanoparticles recovered, and the drug entrapment by the ratio of mass of drug in nanoparticles to mass of drug used in synthesis. The quantitative determination of active component entrapped in nanoparticles is done by extraction of the drug. The polymer dissolution in a suitable solvent (acetonitrile, ethyl acetate, and others) is required, washing steps with distilled water, and purification. The drug concentration of the final suspension can be measured by ultraviolet spectroscopy at defined wavelength (related to the active component) or HPLC. *In-vitro* studies on nanoparticles are dealt in section 2.5.

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2.5 *In vitro* STUDIES ON NANOPARTICLES

2.5.1 *In vitro* release study:

The drug release rates of nanoparticles depend upon,

- Desorption of the surface bound/ adsorbed drug,
- Diffusion through the nanoparticle matrix,
- Diffusion through the polymer wall (in case of nanocapsules),
- Erosion of nanoparticle matrix,
- Combined erosion/diffusion process.

In the case of nanospheres, where the drug is uniformly distributed, the release occurs by diffusion or erosion of the matrix under sink conditions. If the diffusion of the drug is faster than matrix erosion, the mechanism of release is largely controlled by a diffusion process. The rapid initial release or 'burst' is mainly attributed to weakly bound or adsorbed drug to the large surface of nanoparticles (Magenheim et al., 1993). It is evident that the method of incorporation has an effect on release profile. If the drug is loaded by incorporation method, the system has a relatively small burst effect and better sustained release characteristics (Fresta et al., 1995). If the nanoparticle is coated by polymer, the release is then controlled by diffusion of the drug from the core across the polymeric membrane. The membrane coating acts as a barrier to release, therefore, the solubility and diffusivity of drug in polymer membrane becomes determining factor in drug release. Furthermore release rate can also be affected by ionic interaction between the drug and addition of auxiliary ingredients.

In studying *in vitro* drug release from NPs an important issue to consider is the method that can be used to measure drug release (D'Souza and DeLuca 2006).

The release experiments were conducted differently by various methods by researchers.

They include diffusion using artificial or biological membranes, dialysis bag diffusion, reverse dialysis, ultrafiltration, ultracentrifugaion and centrifugal ultrafiltration. Therefore it is not easy to compare the results.

Grabnar et al. (2010) compared in vitro release profiles of budesonide from NPs, which were obtained using four different methods for the assessment of drug release: membrane diffusion technique (dialysis or reverse dialysis) and sample and separate technique (filtration or ultracentrifugation).This study revealed that *in vitro* drug release profiles from NPs obtained by four methods were different. *In vitro* release profiles obtained by filtration, centrifugation

and reverse dialysis exhibited a typical biphasic release phenomenon namely initial burst release and consequently sustained release. The critical points in filtration are the pore size and adsorption of dissolved drug on filter membrane. The problem with centrifugation is that smallest particles are very slow to sediment and further dissolution can occur over this time. The fastest burst release phase was obtained after centrifugation following by filtration and reverse dialysis. The release from NPs determined by dialysis was much slower and showed no burst release. In this method the transport across the membrane was rate limiting, and therefore the true release rate was not measured. Every technique has its own advantages and disadvantages. No clear agreement exists on the suitability of the techniques reported to date for drug release determination from particles in the nanometer size range. Apart from the methods, the release kinetics also depends on the release conditions (sink or non-sink conditions, release medium)

Characterization of the *in vitro* drug release from a colloidal carrier is technically difficult to achieve. This can be attributed to the inability of effective and rapid separation of the particles from the dissolved or released drug in the surrounding medium owing to the very small size of nanocarriers (Dolenc et al., 2009). Dialysis bag technique avoids the problem of NP separation as the NP dispersion is placed in the dialysis bag and immersed into the release medium (LeRay et al., 1994).

2.5.2 *In vitro* cell line studies:

The ability to maintain and manipulate cells in culture is a valuable tool for life science researchers. Cell culture models permit the study of a single cell type. Cell culture models also permit measurement of cell responses to conditions which are more tightly regulated than the complex environment presented by an entire organism.

Actually, *in vitro* cytotoxicity assays with cultured cells are widely used to chemicals including cancer chemotherapeutics, pharmaceuticals, biomaterials, natural toxins, antimicrobial agents and industrial chemicals because they are rapid and economical. These cytotoxicity tests measure the concentration of the substance that damages components, structures or cellular biochemical pathways, and they also allow direct extrapolation of quantitative data to similar *in vivo* situations (Barile, 1994; Ishiyama et al., 1996).

Available methods of assessing proliferation and viability:

To study proliferation, several methods are available. Researchers either detect an antigen which is present in proliferating cells but absent in non-proliferating cell, measure DNA synthesis, or monitor the reducing environment of the cells.

The following is a brief summary of these methods.

2.5.2a Detection of antigens associated with proliferation:

The appearance of several antigens during cell proliferation have been noted. A frequently cited antigen used to monitor proliferation of human cells is the antigen recognized by the monoclonal antibody Ki-67 (Gerdes et al., 1983). Because this antigen is expressed during S, G2, and M phases of the cycle but absent in G0 and G1, this monoclonal antibody is also suitable for examination of the cell cycle (Gerdes et al., 1984). Antibodies directed to the cyclins, proteins which are expressed during specific phases of the cell cycle, also have utility in monitoring cell proliferation. For example, cyclin E is expressed as cells move from the G1 phase to the S phase. (Koff et al., 1999; Dulic et al., 1992).

2.5.2b ^{3}H-Thymidine incorporation:

When supplied to proliferating cells, ^{3}H-thymidine is incorporated into nacently synthesized DNA. While providing an accurate indicator of DNA synthesis, measurement of ^{3}H-thymidine incorporation has several disadvantages. Most notable are the complications of using radioactivity. Quantitation typically involves immunohistochemistry or scintillation counting of labeled cells collected by aspiration upon membrane filters.

2.5.2c Bromodeoxyuridine Incorporation:

Like 3H-thymidine, bromodeoxyuridine is incorporated into nacently synthesized DNA and provides a measurement of cell proliferation (Gratzner, 1982).

2.5.2d Neutral red uptake:

The quantitation of Neutral Red also has utility in measuring cell proliferation. Proliferation assays are performed by culturing cells in microtiter well plates or trays followed by Neutral Red staining. The cells under study may be in suspension or adherent. The absorbance of the Neutral Red stained cells monitored at 550 nm is indicative of the cell number (Kull and Cuatrecasas, 1983).

2.5.2e Tetrazolium Salt Reduction:

The internal environment of proliferating cells is more reduced than that of non-proliferating cells (Mossmann, 1983). This reduced state can be measured using electron acceptors in tetrazolium salts. The most frequently used of these are MTT (3-(4,5-dimethyethiazol-2-yl)-2,5-diphenyltetrazolium bromide), XTT (sodium 3'-[1-phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate, and MTS. MTT is a water soluble tetrazolium salt, which is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria. The formazan product is impermeable to the cell membranes and therefore it accumulates in healthy cells.

The MTT assay was tested for its validity in various cell lines (Fotakis and Timbrell, 2006). Tetrazolium salt reduction, as an indicator of cell growth, has been used in models for screening cytocidal chemical agents (Scudiero et al., 1988) or cell growth promoting factors and cytokines. MTT is best suited for use with adherent cell lines. XTT has the advantage over MTT in that it is soluble in culture medium, and is therefore suitable for use with non-adherent as well as adherent cell lines (Scudiero et al., 1988). MTS is a recently developed, alternative tetrazolium salt which also has utility in cell proliferation assays.

2.5.2f Trypan Blue Exclusion:

Measuring percentage Trypan Blue exclusion can rapidly assess cell viability and confirm whether cell culture conditions are optimal. Cells that are viable exclude Trypan Blue, while cells that have died are stained by this dye. Trypan Blue exclusion measurement is also useful for determining whether cells in culture have escaped breakage or disruption (Wright et al, 1992) and to assess apoptosis (Wiley et al., 1995).

2.5.2g Neutral Red Staining:

Neutral Red stains viable cells. This dye is absorbed by viable cells and is concentrated in the lysosomes. Quantitation of Neutral Red staining therefore has utility in monitoring cytotoxicity assays (Elliot and Auersperg, 1993; Modha et al., 1993).

2.5.2h Crystal Violet Inclusion:

Crystal violet inclusion measurement detects cell lysis. This dye stains viable cells that adhere to their culture vessel. Lysed cells simply fall away from their vessel surface and are not stained by this dye. Crystal violet is used in a standard bioassay for TNF-α. Crystal violet-stained cells are detected by spectrophotometric measurement (Coligan et al., 1996).

2.5.2i ^{51}Cr Release:

Cell death is measured by the quantitation of chromium (^{51}Cr) release from labeled target cells. In this assay, living cells nonspecifically incorporate and retain the radionuclide. As the cells are killed, ^{51}Cr is released into the culture medium and is quantitated by scintillation counting (Wright et al. 1992).

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2.6 PLAN OF WORK

In the present thesis, we have tried to explore the therapeutic benefit using Etoposide as cytotoxic drug and Quercetin Dihydrate as angiogenesis inhibitor by incorporating them separately into PLGA nanoparticles.

The plan of work was proposed to proceed on the following lines:

1. Analytical methods for drug estimation

a) Determination of solubility profile of drugs in different solvents.

b) Construction of standard curves using UV-Visible spectrophotometry for drug estimation in nanoparticles.

c) Construction of standard curves using UV-Visible spectrophotometry for drug estimation in phosphate buffer (pH 7.4).

2. Preparation of PLGA nanoparticles formulation.

a) Nanoprecipitation (solvent diffusion) technique.

3. Optimization

Etoposide (ETN) and Quercetin Dihydrate (QDN) nanoparticles were optimized by using following formulation parameters.

a) Polymer concentration.

b) Surfactant concentration .

c) Organic phase volume.

d) Aqueous phase volume.

e) Theoretical drug loading.

4. Characterization of nanoparticle formulation.

a) Particle size and Size distribution.

b) Surface charge i.e. Zeta potential.

c) Shape and surface morphology by TEM studies.

d) Drug entrapment efficiency.

e) Differential scanning calorimetry.

f) Stability studies of nanoparticles.

5. *In vitro* studies.

a) Drug release studies in pH 7.4 phosphate buffer.

b) Cell cytotoxicity studies on A549 cell line by MTT assay.

6. *In vivo* studies.

a) Radiolabelling of formulations.

b) Radiolabelling efficiency studies.

c) Radiolabelled complex stability studies.

c) Biodistribution studies.

d) Tumour growth inhibitory activity using B16F10 mouse melanoma model.

e) Tumour microvessel density evaluation.

7. Analysis and interpretation of results.