# Chapter - 2

## *Literature Review*

## 2.1. What is 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 survey in 2002 by agency of 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.

Cancer 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; check points are bypassed; mitosis is hijacked; and if further repair mechanisms fail, a malignant cell is born. Further cell replication leads to the formation of tumor (Wootton, 2006).

## 2.2. Angiogenesis

Mammalian cells require oxygen and nutrients for their survival and are located within the diffusion limit for oxygen (100 to 200 μm of blood vessels). The multicellular organisms must recruit new blood vessels to grow beyond this size by vasculogenesis and angiogenesis (Figure 2.1). This process of formation of new blood vessels from the pre-existing one (Angiogenesis) is regulated by a balance between pro and anti-angiogenic molecules (Table 2.1), and is derailed in various diseases, especially cancer. The tumours cannot grow beyond a certain size or metastasize to another organ without blood vessels. Similarly, without an efficient blood supply we may not be able to deliver anti-cancer drugs to all regions of a tumour in effective quantities (Carmeliet and Jain, 2000).

In solid tumors of 1-2$mm^3$, the oxygen and nutrients can reach the center of the tumor by simple diffusion. The non-angiogenic tumors are highly dependent on their microenvironment for oxygen and the supply of nutrients because of their non-functional or non-existent vasculature. When tumors reach 2$mm^3$, a state of cellular hypoxia begins, initiating angiogenesis. Angiogenesis is regulated by a fine balance of activators and inhibitors (Bergers and Benjamin, 2003). In the angiogenesis process, five phases can be distinguished: 1. endothelial cell activation, 2. basement membrane degradation, 3. endothelial cell migration, 4. vessel formation, and 5. angiogenic remodeling. Hypoxia increases cellular hypoxia inducible factor (HIF) transcription, leading to upregulation of pro-angiogenic proteins such as vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) or tumor necrosis factor-α (TNF-α) (Carmeliet, 2000). Activated endothelial cells express the dimeric transmembrane integrin $\alpha_v\beta_3$, which interacts with extracellular matrix proteins such as vibronectin and

fibronectin and regulates the migration of the endothelial cell through the extracellular matrix during vessel formation (Avraamides et al., 2008). The activated endothelial cells synthesize proteolytic enzymes, such as matrix metalloproteinases, which degrade the basement membrane and the extracellular matrix. The inner layer of endothelial cells undergoes apoptosis leading to the formation of the vessel lumen. Immature vasculature undergoes extensive remodeling during which the vessels are stabilized by pericytes and smooth-muscle cells. This step is often incomplete resulting in irregular shaped, dilated and tortuous tumor blood vessels (Stollman et al., 2009). This ability of tumors development from a non-angiogenic to angiogenic phenotype (called the "angiogenic switch") is central for progression of cancer and allows the distribution of cancer cells throughout the body, leading to metastasis (Bergers and Benjamin, 2003; Naumov et a., 2006).

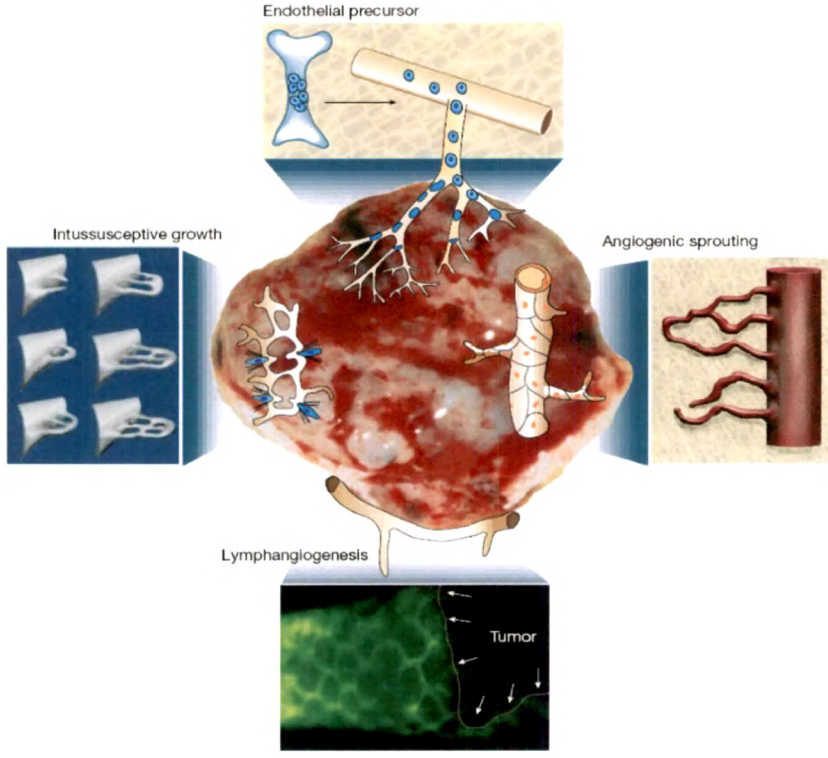



**Figure 2.1.** Cellular mechanisms of tumour (lymph) angiogenesis. Tumour vessels grow by various mechanisms: (A) the host vascular network expands by budding of endothelial sprouts or formation of bridges (angiogenesis); (B) tumour vessels remodel and expand by the insertion of interstitial tissue columns into the lumen of pre-existing vessels (intussusception); and (C) endothelial cell precursors (angioblasts) home from the bone marrow or peripheral blood into tumours and contribute to the endothelial lining of tumour vessels (vasculogenesis). Lymphatic vessels around tumours drain the interstitial fluid and provide a gateway for metastasizing tumour cells (Adapted from Leu et al., 2000).

**Table 2.1.** Endogenous angiogenic and anti-angiogenic factors (Makrilia et al., 2009)

| Angiogenic factors | Anti-angiogenic factors |
|---|---|
| Vascular endothelial growth factor | Angiostatin |
| Angiopoietins | Endostatin |
| Acidic and basic fibroblastic growth factors | Thrombospondin-1/2 |
| Platelet-derived endothelial cell growth factor | Vasostatin |
| Transforming growth factor-a/b | Platelet-associated platelet factor-4 |
| Tumour necrosis factor-a | Osteopontin |
| Epidermal growth factor | Tissue inhibitor metalloproteinases (TIMP) |
| Cyclooxygenase-2 | Interleukin-12 |
| Interleukin-6/8 | |

### 2.3. Tumour angiogenesis: Therapeutic implications

Anti-angiogenic therapy suppresses the cancer indirectly, by depriving cells of nutrients and oxygen. The excess production of pro-angiogenic molecules and/or diminished production of anti-angiogenic molecules results in tumour vessels and tumour microenvironment abnormalities. It is possible that these factors interfere with the delivery of therapeutic drugs, by rendering tumour cells resistant to both radiation and cytotoxic therapy, induce genetic stability and select for malignant cells with increased metastatic potential and compromise the cytotoxic functions of immune cells (Jain, 1988). It has been proposed by Jain et al. that if one can restore the balance of pro- and anti-angiogenic factors, the vasculature might revert back to a more "normal" state, leading to deeper penetration of therapeutic molecules into tumour tissue (Jain et al., 2007). Anti-angiogenic treatment strategies are supposed to have advantages compared to conventional cytotoxic chemotherapy, directed against malignant tumour cells. In particular, anti-angiogenic molecules are not restricted to a specific histologic tumour entity, since all solid tumours depend on angiogenesis. Moreover, the angiogenesis in adults is induced only under certain conditions such as wound healing or the reproductive ovarian cycle. Therefore, anti-angiogenic therapy is a highly selective option, promising less serious side effects. In addition, vascular endothelial cells have genetically stable MHC expression on the surface, which will not be down-regulated, in contrast to the surface of tumour cells. Thus, the endothelial cell, as a target, is suggested to be less prone to developing drug resistance (Poon et al., 2001; Poon et al., 2003).

### 2.4. Vascular targeting therapy

The vascular targeting therapy aims at destroying the existing vasculature of a tumour. The three classes of vascular targeting therapy have yet been proposed. Firstly, combretastatin derivatives staunch blood flow and inhibit tumour growth through the disruption of the tubulin cytoskeleton of endothelial cells which leads to thrombosis of the vasculature (Hori et al., 2002). Another approach is the targeted gene delivery to the neovasculature. This is achieved with cationic nanoparticles bound to an integrin $\alpha_v\beta_3$-

directed ligand that deliver a mutant gene to tumour vessels (Hood et al., 2002). The third approach is cationic liposome-based vascular targeting therapy, which relies on a selective propensity for targeting activated tumour endothelium. More specifically, angiogenic endothelial cells express negative charge therefore cationic liposomes can actively bind to them and deliver cytotoxic drugs (Eichhorn et al., 2004).

### 2.5. Drug targeting to solid tumors

The tumor microenvironment is one of many areas which are studied to design new cancer therapies. More precisely, the knowledge and the understanding of the tumor microenvironment allow researchers to elaborate different therapeutic strategies, based on numerous differences compared with normal tissue including vascular abnormalities, oxygenation, perfusion, pH and metabolic states. The differences in terms of morphology of tumor vasculature and the pH will be more relevant characteristics for the design of nanocarriers as tumor targeted drug delivery systems.

### 2.6. Passive targeting

The passive targeting consists of the transport of nanocarriers through leaky tumor capillary fenestrations into the tumor interstitium and cells by convection or passive diffusion (Figure 2.2A) (Haley and Frenkel, 2008). The enhanced permeation and retention (EPR) effect is now becoming the gold standard in cancer-targeting drug designing. All nanocarriers use the EPR effect as a guiding principle. Moreover, for almost all rapidly growing solid tumors the EPR effect is applicable (Maeda et al., 2009). The EPR effect can be observed in almost all human cancers with the exception of hypovascular tumors such as prostate cancer or pancreatic cancer (Maeda et al., 2001; Unezaki et al., 1996). The EPR effect will be optimal if nanocarriers can evade immune surveillance and circulate for a long period. Very high concentrations of drug-loaded nanocarriers can be achieved at the tumor site, for instance 10-50-fold higher than in normal tissue within 1-2 days (Iyer et al., 2006). For this, at least three properties of nanocarriers are particularly important. (1) The ideal nanocarriers size should be between 10 and 100 nm. Indeed, for efficient extravasation from the fenestrations in leaky vasculature, nanocarriers should be much less than 400 nm. On the other hand, to avoid the filtration by the kidneys, nanocarriers need to be larger than 10 nm; and to avoid the specific capture by the liver, nanocarriers need to be smaller than 100 nm. (2) The charge of the nanoparticles should be neutral or anionic for efficient evasion of the renal elimination. (3) The nanocarriers must be hidden from the reticuloendothelial system, which destroys any foreign material through opsonisation followed by phagocytosis (Malam et al., 2009; Gullotti et al., 2009). However, to reach passively the tumor, some limitations exist. (a) The passive targeting depends on the degree of tumor vascularisation and angiogenesis (Bae, 2009). Thus extravasation of nanocarriers will vary with tumor types and anatomical sites. (b) The high interstitial fluid pressure of solid tumors avoids successful uptake and homogenous distribution of drugs in the tumor (Heldin et al., 2004). The high interstitial fluid pressure of tumors associated with

the poor lymphatic drainage leads to more retention of larger and long-circulating nanocarriers (100 nm) in the tumor, whereas smaller molecules easily diffuse (Pirollo and Chang, 2008) (Figure 2.2A.2).

**2.7. Active targeting**

In active targeting, the targeting ligands are attached at the surface of the nanocarrier for binding to appropriate receptors which over-expressed at the target site (Figure 2.2B). The ligand chosen bind to a receptor over-expressed by tumor cells or tumor vasculature and not expressed by normal cells. The targeted receptors should be expressed homogeneously on all targeted cells. Targeting ligands are either monoclonal antibodies (mAbs) and antibody fragments or non-antibody ligands (peptidic or not). The binding affinity of the ligands influences the tumor penetration because of the "binding-site barrier." For targets in which cells are readily accessible, typically the tumor vasculature, because of the dynamic flow environment of the bloodstream, high affinity binding appears to be preferable (Adams et al., 2001; Gosk et al., 2008). Various anticancer therapeutics, grouped under the name "ligand targeted therapeutics," are divided into different classes based on the approach of drug delivery (Allen, 2002). The common basic principle of all these therapeutics is the specific delivery of drugs to cancer cells. Antibodies (monoclonal antibody or fragments) target a specific receptor, interfering with signal-transduction pathways, regulating proto-oncogenes involved in cancer cells proliferation-such as trastuzumab (anti-ERBB2, Herceptin®), bevacizumab (anti-VEGF, Avastin®) or etaracizumab, a humanized anti-$\alpha_v\beta_3$ antibody (Abegrin). In this case, the active molecule plays the role of both targeting ligand and drug. Antibodies (or fragments) may only play the role of targeting ligand when they are coupled with therapeutic molecules. $^{90}$yttrium-ibritumomab tiuxetan (Zevalin®), directed against anti-CD-20, was the first radioimmunotherapeutic received for clinical approval (Wiseman et al., 2001). The first immunotoxin approved in clinical was denileukin diftitox (Ontak®), an interleukin (IL)-2-diphteria toxin fusion protein (Duvic et al., 2002). The only immunoconjugate to receive clinical approval is gemtuzumab ozogamicin (Mylotarg®) (Jurcic, 2001). Immuno-nanocarriers use a different approach: cytotoxic drug is encapsulated into a nanocarrier and antibodies (or fragments), the targeting ligands, are coupled to the particle surface. Finally, for targeted nanocarriers, antibodies are replaced by molecule (peptidic or not) binding to specific receptors. In the active targeting strategy, two cellular targets can be distinguished: (i) the targeting of cancer cell (Figure 2.2B.1) and (ii) the targeting of tumor endothelium (Fig. 2.2B.2).

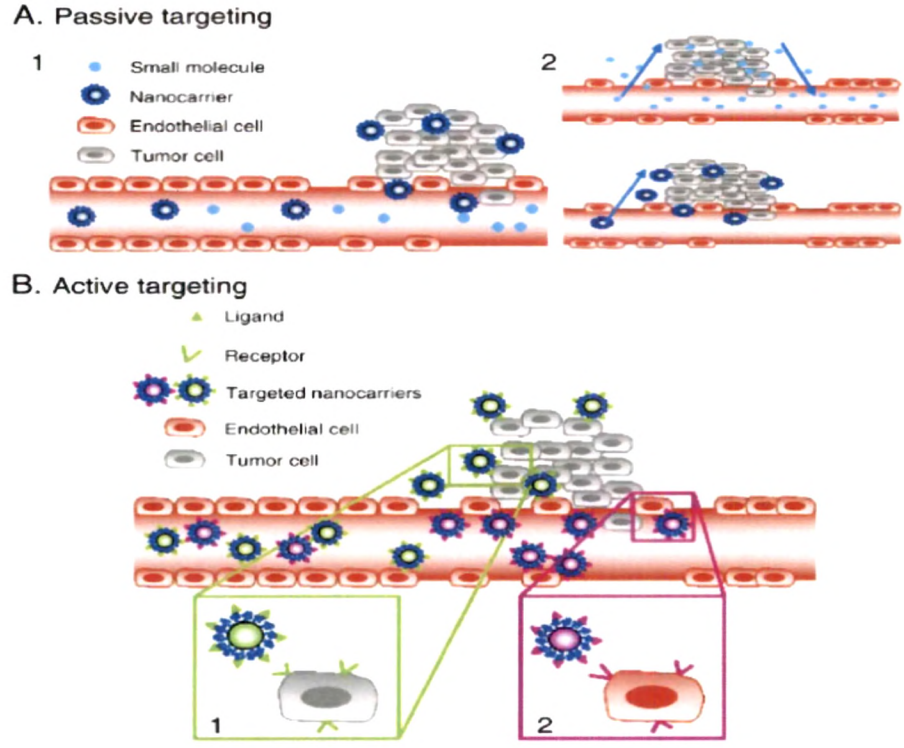



**Figure 2.2. A.** Passive targeting of nanocarriers. (1) Nanocarriers reach tumors selectively through the leaky vasculature surrounding the tumors. (2) Schematic representation of the influence of the size for retention in the tumor tissue. Drugs alone diffuse freely in and out the tumor blood vessels because of their small size and thus their effective concentrations in the tumor decrease rapidly. By contrast, drug-loaded nanocarriers cannot diffuse back into the blood stream because of their large size, resulting in progressive accumulation: the Enhanced permeation and retention (EPR) effect. **B.** Active targeting strategies. Ligands grafted at the surface of nanocarriers bind to receptors (over)expressed by (1) cancer cells or (2) angiogenic endothelial cells. (Figure adapted from Danhier et al., 2010)

### 2.8. Cancer cell targeting

The objective of active targeting *via* internalization-prone cell-surface receptors, over-expressed by cancer cells, is to improve the cellular uptake of the nanocarriers. Thus, the active targeting is particularly attractive for the intracellular delivery of macromolecular drugs, such as DNA, siRNA and proteins. The enhanced cellular internalization rather than an increased tumor accumulation is responsible of the anti-tumor efficacy of actively targeted nanocarriers. This is the base of the design of delivery systems targeted to endocytosis-prone surface receptors (Kirpotin et al., 2006). The ability of the nanocarrier to be internalized after binding to target cell is thus an important criterion in the selection of proper targeting ligands (Cho et al., 2008). In this strategy, ligand targeted nanocarriers will result in direct cell kill, including cytotoxicity against cells

that are at the tumor periphery and are independent on the tumor vasculature (Pastorino et al., 2006). The more studied internalization-prone receptors are:

**(a)** The transferrin receptor. Transferrin, a serum glycoprotein, transports iron through the blood and into cells by binding to the transferring receptor and subsequently being internalized *via* receptor-mediated endocytosis. The transferrin receptor is a vital protein involved in iron homeostasis and the regulation of cell growth. The high levels of expression of transferrin receptor in cancer cells, which may be up to 100-fold higher than the average expression of normal cells, its extracellular accessibility, its ability to internalize and its central role in the cellular pathology of human cancer, make this receptor an attractive target for cancer therapy (Cho et al., 2008; Daniels et al., 2006).

**(b)** The folate receptor is a well-known tumor marker that binds to the vitamin folic acid. Folic acid is required in one carbon metabolic reactions and consequently, is essential for the synthesis of nucleotide bases. The alpha isoform, folate receptor-α is overexpressed on 40% of human cancers. In contrast, folate-receptor-β is expressed on activated macrophages and also on the surfaces of malignant cells of hematopoietic origin (Low and Kularatne, 2009).

**(c)** Glycoproteins. Lectins are proteins of non-immunological origin which are able to recognize and bind to carbohydrate moieties attached to glycoproteins expressed on cell surface. Cancer cells often express different glycoproteins compared to normal cells. Lectins interaction with certain carbohydrate is very specific. Lectins can be incorporated into nanoparticles as targeting moieties that are directed to cell-surface carbohydrates (direct lectin targeting) and carbohydrates moieties can be coupled to nanoparticles to target lectins (reverse lectin targeting). The use of lectins and neoglycoconjugates for direct or reverse targeting strategies is a traditional approach of colon drug targeting (Minko, 2004).

**(d)** The Epidermal growth factor receptor (EGFR) is a member of the ErbB family, a family of tyrosine kinase receptors. Its activation stimulates key processes involved in tumor growth and progression, including proliferation, angiogenesis, invasion and metastasis. EGFR is frequently over-expressed in many of cancer, especially in breast cancer, has also been found to play a significant role in the progression of several human malignancies. Human epidermal receptor-2 (HER-2) is reported to be expressed in 14-91% of patients with breast cancer (Acharya et al., 2009; Scaltriti and Baselga, 2006). EGFR is expressed or over-expressed in a variety of solid tumors, including colorectal cancer, non-small cell lung cancer and squamous cell carcinoma of the head and neck, as well as ovarian, kidney, pancreatic, and prostate cancer (Lurje and Lenz, 2009).

### 2.9. Tumor endothelial cell targeting

Destruction of the solid tumors endothelium can result in the death of tumor cells induced by the lack of oxygen and nutrients. In 1971, Judah Folkman suggested that the tumor growth might be inhibited by preventing tumors from recruiting new blood vessels (Folkman, 1971). This observation is the base of the design of nanomedicines actively targeted to tumor endothelial cells (Lammers et al., 2008). By attacking the

growth of the blood supply, the size and metastatic capabilities of tumors can be regulated. Thus, the ligand-targeted nanocarriers bind to and kill angiogenic blood vessels and indirectly, the tumor cells that these vessels support, mainly in the tumor core. The advantages of the tumoral endothelium targeting are: (1) there is no need of extravasation of nanocarriers to arrive to their targeted site, (2) the binding to their receptors is directly possible after intravenous injection, (3) the potential risk of emerging resistance is decreased because of the genetically stability of endothelial cells as compared to tumor cells, and (4) most of endothelial cells markers are expressed irrespective of the tumor type, involving an ubiquitous approach and an eventual broad application spectrum (Gosk et al., 2008). The main targets of the tumoral endothelium include:

**(a)** The vascular endothelial growth factors (VEGF) and their receptors, VEGFR-1 and VEGFR-2, mediate vital functions in tumor angiogenesis and neovascularization (Shadidi Sioud, 2003). The tumor hypoxia and oncogenes upregulate VEGF levels in the tumor cells which results in upregulation of VEGF receptors on tumor endothelial cells. Two major approaches to target angiogenesis *via* the VEGF have been studied: (i) targeting VEGFR-2 to decrease VEGF binding and induce an endocytotic pathway and (ii) targeting VEGF to inhibit ligand binding to VEGFR-2 (Byrne et al., 2008; Carmeliet, 2005).

**(b)** The $\alpha_v\beta_3$ integrin is an endothelial cell receptor for extracellular matrix proteins which includes fibrinogen (fibrin), vibronectin, thrombospondin, osteopontin and fibronectin (Desgrosellier and Cheresh, 2010). The $\alpha_v\beta_3$ integrin is highly expressed on neovascular endothelial cells but poorly expressed in resting endothelial cells and most normal organs, and is important in the calcium dependent signalling pathway leading to endothelial cell migration (Byrne et al., 2008). Cyclic or linear derivatives of RGD (Arg-Gly-Asp) oligopeptides are the most studied peptides which bind to endothelial $\alpha_v\beta_3$ integrins. The $\alpha_v\beta_3$ integrin is upregulated in both tumor cells and angiogenic endothelial cells (Desgrosellier and Cheresh, 2010).

**(c)** Vascular cell adhesion molecule-1 (VCAM-1) is an immunoglobulin like transmembrane glycoprotein that is expressed on the surface of endothelial tumor cells. VCAM-1 induces the cell to cell adhesion, a key step in the angiogenesis process. Over-expression of VCAM-1 is found in various cancers, including leukemia, lung and breast cancer, melanoma, renal cell carcinoma, gastric cancer and nephroblastoma (Dienst et al., 2005).

**(d)** The matrix metalloproteinases (MMPs) are a family of zinc dependent endopeptidases. MMPs degrade the extracellular matrix, playing an essential role in angiogenesis and metastasis more particularly in endothelial cell invasion and migration, in the formation of capillary tubes and in the recruitment of accessory cells. Membrane type 1 matrix metalloproteinase (MT1-MMP) is expressed on endothelial tumor cells, including malignancies of lung; gastric, colon and cervical carcinomas; gliomas and melanomas (Genis et al., 2006). Aminopeptidase N/CD13, a

metalloproteinase that removes amino-acids from unblocked N-terminal segments of peptides or proteins, is an endothelial cell-surface receptor involved in tumor-cell invasion, extracellular matrix degradation by tumor cells and tumor metastasis *in vitro* and *in vivo* (Saiki et al., 1993). NGR (Asn-Gly-Arg) peptide is reported to bind to the aminopeptidase (Pasqualini et al., 2000).

### 2.10. Neuropilins

Neuropilin-1 (NRP1) was originally identified as the antigen of a specific monoclonal antibody called A5, raised against neuronal cell surface proteins presumed to be involved in neuronal recognition between the visual centres and the optic nerve fibres of Xenopus laevis (Takagi et al., 1991). It was subsequently named neuropilin because A5 bound to the superficial neuropile of the tadpole optic tectum (Fujisawa et al., 1995). Further work demonstrated an essential role of NRP1 in development of the embryonic nervous and cardiovascular systems (Gu et al., 2003; Kawasaki et al., 1999). NRP1 and the structurally-related molecule, NRP2, are receptors both for class 3 semaphorins, a family of secreted polypeptides with key roles in axonal guidance, and for various members of the VEGF family of angiogenic cytokines, but are thought to transduce functional responses only when co-expressed with other receptors: plexins in the case of semaphorins and VEGFR2 for VEGFs. NRPs are also highly expressed in diverse tumour cell lines and human neoplasms and have been implicated in tumour growth and vascularisation *in vivo* (Gagnon et al., 2000; Klagsbrun et al., 2002). More recently, NRP1 was also found to be a novel mediator of the primary immune response (Bruder et al., 2004; Tordjman et al., 2002). These findings suggest that NRPs are multi-functional co-receptors essential for neuronal and cardiovascular development, and potentially with additional roles in other physiological and disease-related settings. However, despite the wealth of information regarding the likely biological functions of these molecules, many aspects of the regulation of cellular function *via* NRPs remain uncertain, and little is known concerning the molecular mechanisms through which NRPs mediate the functions of their various ligands in different cell types.

Figure 2.3 shows the protein structure of full-length and soluble neuropilins. Full-length NRPs are composed of two complement binding domains (CUB), two blood coagulation factors V and VIII homology domains (FV/VIII), one meprin-A5-phosphatase domain (MAM), a transmembrane domain and a cytoplasmic domain containing a PDZ binding domain (for NRP-1 and NRP-2a). Soluble forms do not include the MAM, transmembrane and cytoplasmic domains. Percentage homology between NRP1 and NRP2a and between NRP2a and NRP2b are displayed on the Figure 2.3. On the top part are represented the genomic DNA structure of NRP1 as well as the cDNA composed of 17 exons.

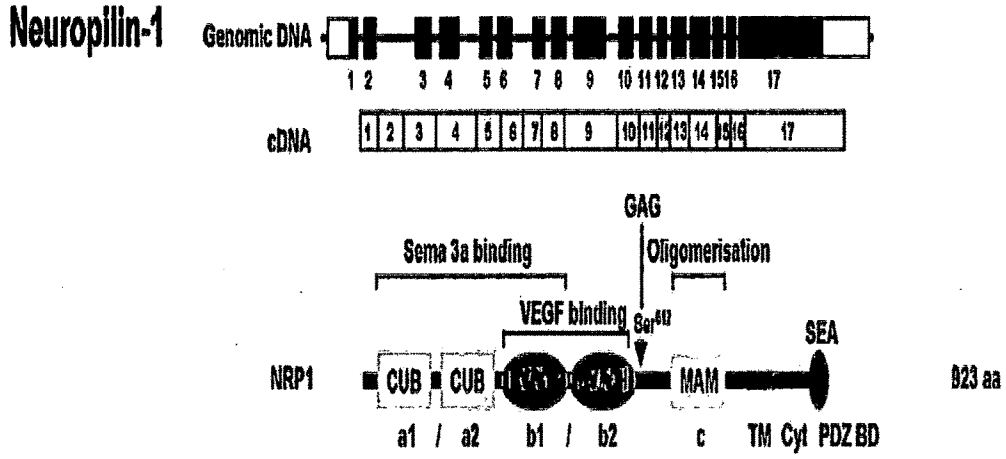



**Figure 2.3.** Neuropilin-1 domain structure homology (Pellet-Many et al., 2008)

The mechanism of NRPs is shown in Figure 2.4. In sensory neurons, NRP1 can complex with plexin A1 or A2, or with L1-CAM. L1 CAM contains six Immunoglobulin (Ig)-like domains (red ellipses) and five fibronectin type III domains (green squares) in its extracellular region. The L1 intracellular domain associates with ankyrin (rounded brown rectangle), which may link L1 with spectrin (chain of yellow ellipses) and actin (red chain of small red circles), and is implicated in mediating effects of Sema 3A on the cytoskeleton. Plexin A1 activity is regulated by the rho-like GTPases Rnd and its antagonist RhoD (respectively, beige and light blue circles), though how NRP1 regulates this pathway is unclear; the inhibitory effect of RhoD on plexin A1 activity is represented by a red barred line. In endothelial cells VEGF-A165 binds to NRP1 and promotes complex formation. The NRP1 carboxy-terminal PDZ domain binding motif associates with the PDZ protein synectin and this is thought to be important for the role of NRP1 in VEGF-A signalling and function mediated *via* VEGFR2. In some contexts, VEGF-A165 and Sema 3A may also competitively bind to NRP1 resulting either in inhibition of Sema 3A binding and downstream signalling, or potentially the converse (indicated by a red bars). NRP1/VEGFR2 complex formation is implicated in optimal activation (indicated by +), *via* a mechanism that is still unclear (?), of VEGFR2 signalling *via* phospholipase C-gamma (PLC-γ), leading to activation of PKC and ERK1/2, and PI3K, mediating activation of the serine-threonine kinase, Akt/PKB (Pellet-Many et al., 2008).

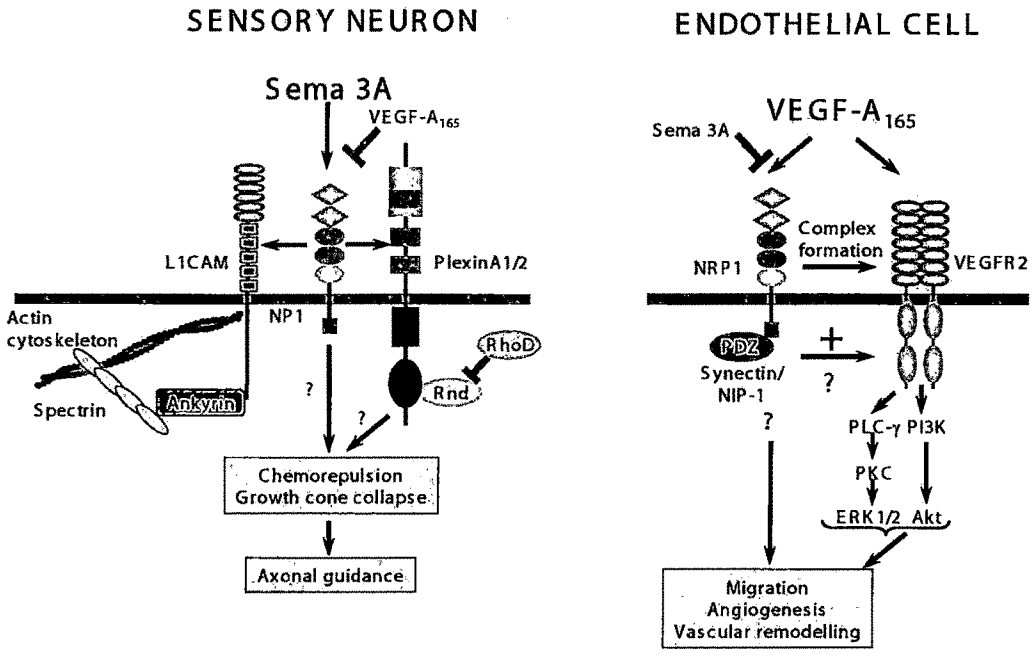



**Figure 2.4.** Mechanisms of NRP action (Figure adapted from Pellet-Many et al., 2008).

NRP1 and NRP2 are expressed by a wide variety of human tumour cell lines and diverse human neoplasms (Bielenberg et al., 2006; Ellis, 2006), and are implicated in mediating effects of VEGF and Semaphorins on the proliferation, survival and migration of cancer cells (Bachelder et al., 2001; Chabbert-de et al., 2006). Table 2.2 summarises the expression of NRPs and other VEGF receptors in a panel of representative carcinoma cells. Over-expression of NRP1 in Dunning rat prostate AT2.1 carcinoma cells increased tumour growth *in vivo* (Miao et al., 2000), while NRP1 knockdown using siRNA inhibited breast carcinoma cell migration (Bachelder et al., 2003), and a peptide targeted to the VEGF binding site of NRP1 induced breast tumour cell apoptosis (Barr et al., 2005). NRP1 is expressed in patient specimens from lung, breast, prostate, pancreatic and colon carcinomas, but not in corresponding normal epithelial tissues (Fukahi et al., 2004). NRP1 has also been found in several other tumours including melanoma (Straume and Akslen, 2003), astrocytoma (Broholm and Laursen, 2004) and neuroblastoma (Fakhari et al., 2002). NRP2 expression was reported in lung cancer (Lantuejoul et al., 2003), neuroblastoma (Fakhari et al., 2002), pancreatic cancer (Cohen et al., 2002), osteosarcoma (Handa et al., 2000) and bladder cancer (Sanchez-Carbayo et al., 2003). It has been suggested that NRP1 is more prevalently expressed in carcinomas (mainly of epithelial origin), whereas NRP2 may be more frequently expressed in non-carcinoma neoplasms such as melanomas, leukaemias and neuroblastomas (Ellis, 2006). However, as Table 2.2 indicates there is no sharp distinction between the types of neoplasms expressing NRPs 1 and 2 (Marcus et al., 2005), and often they are co-

expressed. Furthermore, different cell lines derived from the same tumour types, such as glioma (Rieger et al., 2003), may exhibit divergent patterns of NRP1 and NRP2 expression.

**Table 2.2.** Neuropilins and VEGFRs expression in tumour cells

| | NRP1 | NRP2 | VEGFR2 | VEGFR1 | VEGFR3 |
|---|---|---|---|---|---|
| A549 (lung) | ++ | + | - | - | - |
| MDA-MB-231 (breast) | + | ? | - | ++ | - |
| MCF-7 (breast) | + | ? | - | ++ | - |
| ACHN (kidney) | + | ++ | - | - | - |
| SKOV-3 (ovary) | + | + | - | - | - |
| DU145 (prostate) | ++ | - | - | - | - |
| RT112/84 (bladder) | + | ? | - | - | - |
| BxPC-3 (pancreas) | + | ? | - | - | - |
| SK-MEL-5 (melanoma) | +/- | ++ | - | - | - |
| MDA-MB-435 (melanoma) | + | ++ | - | - | - |
| SK-N-AS (neuroblastoma) | + | - | - | - | ND |
| SK-N-DZ (neuroblastoma) | + | - | - | - | ND |
| U87 MG (glioma) | ++ | + | ND | ND | ND |
| Astrocytoma(GBM) | + | ? | - | - | - |
| Leukaemia (AML, various) | + | + | - | +/- | +/- |
| Leukaemia (CML, various) | + | + | +/- | +/- | - |

AML, acute myeloid leukaemia cell lines; CML, chronic myeloid leukaemia cell lines; ND, not determined; ?, very low or uncertain expression; +/-, low expression or no expression in other; +, moderate expression; ++, strong expression.

### 2.11. Immunoliposomes

The demand for increased specificity of anticancer agents to target tumors has resulted in numerous strategies, including oncogene selective inhibitors, gene and antisense therapies, and monoclonal antibodies (Gregoriadis, 1976), as well as combinations thereof. Although many of the regimens are already in use, the need for more effective targeted drug delivery methods requires further modification of anticancer therapies. Improved delivery of drug-carrying immunoliposomes is one approach to this problem. Park et al. (Park et al., 2002) reported the feasibility of this system as a potential anticancer regimen.

Cytotoxic drug incorporation into liposomes has been reported since the mid-1970s. These early reports laid the groundwork for selecting therapeutic agents amenable to incorporation by liposomes, as well as determining optimal liposome size and net charge required for effective drug delivery (Gregoriadis, 1976). Previous reports also noted the problems associated with liposome-mediated drug delivery; liposomes were removed from circulation by fixed macrophages in the RES, particularly in the liver and spleen. Although, the first-generation liposome-incorporated drugs may be effective in macrophage-related diseases, particularly in the liver and spleen, many other tumors would require other drug-targeting mechanisms. Later studies refined many of the considerations required for effective liposome-mediated drug delivery (Drummond et al., 1999). Today, drugs of various chemical properties have been successfully packaged, including hydrophilic drugs such as N-(phosphonoacetyl)-L-aspartate, hydrophobic drugs such as paclitaxel, and amphipathic drugs such as doxorubicin.

The mechanism by which sterically stabilized liposomes are thought to decrease RES-mediated uptake is that the stabilizer occupies the space immediately adjacent to the liposomal surface, excluding other macromolecules from this space. Therefore, access to and binding of blood plasma opsonins to the liposome surface are hindered, preventing interactions with RES macrophages. Although, the sterically stabilized liposomes prolong circulation time and decrease liposomal uptake by the RES, they do not actively target the liposome to the tumor. One effective means of targeting tumors would be *via* conjugation of antitumor antibodies or portions of antibodies to liposomes (immunoliposomes). In this approach, it has become apparent that many factors must be taken into consideration, including proper choice of target antigen, antibody function, and antibody liposome linkage (Park et al., 1997). Thus, tumor antigens must be identified, and the biological response of a given antibody toward the tumor antigen determined. Detection of tumor-specific antigens has proven difficult because most tumors do not express unique antigens. Rather, they can express the same antigens as normal tissue but in greater quantities compared with normal cells. In addition, many tumors do not over-express the antigens homogeneously throughout the primary tumor or may not express them in metastases. Some antigens may be shed or secreted, leading to potentially high levels of soluble antigen that could interfere with immunotherapies. In spite of the potential difficulties, the successful use of monoclonal antibodies such as herceptin in immunotherapy suggests that immunoliposomes may represent a viable approach.

Chimeric or humanized monoclonal antibodies can reduce the host response against the therapeutic antibody (Winter et al., 1993). Removing the Fc portion of the IgG antibody can also reduce antigenicity. In addition, the cellular internalization of antibodies increases the efficacy of drug delivery, presumably by inducing tumor cells to endocytose immunoliposomes. This is what happens with the HER2+targeted immunoliposomes, which distribute within solid tumors and not simply in the

extracellular space surrounding the tumor blood vessels (Park et al., 1997; Kirpotin et al., 1997).

Therefore, the effective targeted drug delivery using immunoliposomes requires considerations of liposome, antibody, and the chemotherapeutic agent, as well as their interactions with each other and the targeted cell. These considerations are summarized by Park et al. (Park et al., 1997) and in Table 2.3. In their report, Park et al. (Park et al., 2002) used sterically stabilized liposomes, a humanized or completely human anti-HER2 Fab' fragment, and doxorubicin. Doxorubicin has a relatively broad activity against a variety of tumors, can be efficiently loaded into and effectively delivered by the liposomal carrier. Park et al. (Park et al., 2002) demonstrate that the doxorubicin-containing anti-HER2 immunoliposome is more effective than any portion of these components in reducing growth of HER2-over-expressing breast cancer cells that were s.c. implanted into nude mice. These results reveal that immunoliposomes can overcome the potential barriers for delivery into tumor tissues, suggesting that with proper construction of the Fab' fragment of a properly chosen monoclonal antibody, proper liposome composition, and proper drug loading, immunoliposomes can be effective anticancer agents. However, several questions must be addressed. For example, are anti-HER2 immunoliposomes also effective in orthotopically, rather than s.c., implanted HER2-overexpressing breast tumors? Can immunoliposomes (anti-HER2 or others) effectively treat metastatic lesions, because small lesions (□1.2mm) appear to be avascular (Folkman, 1971; Folkman, 1990)? Therefore, micrometastases may not be particularly amenable to treatment with i.v. administered liposomal drugs that require extravasation for activity. As the use of immunoliposomes as anticancer agents approaches clinical trials, more questions arise. What are the immediate and long-term effects of immunoliposome administration in patients? Will immunoliposomes, similar to monoclonal antibodies, also be able to overcome potential barriers such as tumor heterogeneity? Will immunoliposomes be effective in combination therapies? These and other questions await further studies.

**Table 2.3.** Components of Immunoliposomes design (Adapted from Park et al., 1997)

| Component | Considerations for optimal design |
|---|---|
| Target antigen | Homogeneously overexpressed<br>Vital to tumor progression<br>Does not solubilize into circulation |
| Antibody | Humanized or human mAb fragment<br>Efficiently endocytosed<br>Intrinsic antitumor activity |
| Linkage | Ab covalently attached to hydrophobic anchor<br>Specific sites on Ab and liposome; avoids steric hindrance |
| Liposome | Stable as intact construct *in vivo*<br>Long-circulating<br>Selective extravasation in tumors; small diameter to improve tumor penetration |
| Drug | Efficient and high-capacity encapsulation<br>Increased efficacy with bystander effect<br>Anticancer effect particularly suited to target cell population<br>Cytotoxicity enhanced by binding of mAb |

## 2.12. Actively tumor targeted liposomes under preclinical and clinical trials

The active targeting strategy consists of grafting a targeting ligand at the surface of nanocarriers (liposomes) to provide an enhanced selectivity and thus efficacy, as compared to the passive targeting. Although many authors report the evidence of this strategy in preclinical models, until now only two clinical trials have been conducted for ligand conjugated liposomes (Table 2.4), the GAH-targeted doxorubicin-containing immunoliposomes (MCC-465) (Matsumura et al., 2004) and the transferrin-targeted oxaliplatin containing liposomes (Suzuki et al., 2008). On the other hand, a much larger number of preclinical studies are published, using various nanomedicines (liposomes) and targeting ligands (Table 2.4). The type of conjugation strategies used in the preparation of these actively targeted liposomes along with their liposomal composition was shown in Table 2.5.

## 2.13. Recent activities of immunoliposome preparation

### 2.13.1. Single chain antibodies (ScFv)

Recombinant antibody (rAb) fragments are becoming popular therapeutic alternatives to full length monoclonal Abs since they are smaller, possess different properties that are advantageous in certain medical applications, can be produced more economically and are easily amendable to genetic manipulation. Single-chain variable fragment (scFv) Abs are one of the most popular rAb format as they have been engineered into larger, multivalent, bi-specific and conjugated forms for many clinical applications (Weisser and Hall, 2009).

scFv Abs (26-28 kDa) are composed of $V_H$ and $V_L$ chains that are joined *via* a flexible peptide linker (Maynard and Georgiou, 2000). The first scFv molecules were developed independently by Huston et al. (Huston et al., 1988) and Bird et al. (Bird et al., 1988) and represent the smallest functional $V_H$-$V_L$ domains of an Ab necessary for high-affinity binding of antigen. Originally derived from genes isolated from murine hybridoma cell lines, an scFv is capable of binding its target antigens with an affinity similar to that of the parent mAb (Bird et al., 1988). Peptide linkers that join the $V_H$ and $V_L$ chains usually vary from 10 to 25 amino acids in length and typically include hydrophilic amino acids; the most common linker is the decapentapeptide $(Gly4Ser)_3$. The easiest constructs to engineer are noncovalent diabody, triabody and tetrabody molecules that assemble according to changes in the linker length (Todorovska et al., 2001). scFvs are predominantly monomeric when the $V_H$ and $V_L$ domains are joined by a linker of 12 or more amino acids. However, scFvs with a linker length of three to 12 residues cannot fold into a functional Fv domains and instead associate with a second scFv molecule to form a dimer (diabody, ~60 kDa) (Holliger et al., 1993) due to pairing of the $V_H$ of one chain to the $V_L$ of another. Furthermore, reducing the linker length to 3 amino acids or less can force scFv association into trimers (triabodies ~90 kDa) (Iliades et al., 1997) or tetramers (tetrabodies ~120 kDa) (Dolezal et al., 2003). Another diabody format, a cysteine-modified diabody (Cys-diabody), has been engineered *via* the introduction of cysteine residues at specific locations for improved stability. Cys-diabodies have the same antigen binding as the non-covalent diabodies with the advantage of being able to be chemically modified (e.g. with a radiometal) following disulfide bond reduction (Olafsen et al., 2004).

In general, monovalent Ab fragments such as scFv, dsFv ($V_H$ and $V_L$ chains joined by disulfide bond) and Fv have a low functional affinity and a short *in vivo* half-life, due to their small size and valency, properties of which are detrimental to some therapeutic applications. However, because rAb fragments are easily and cost effectively expressed, and are easily subjected to genetic engineering, they remain attractive therapeutic candidates. As a result, Ab engineering endeavours have generated various multi-functional and multivalent scFv-based fragments that have proven to be superior therapeutic reagents (e.g. scFv-Fc, scFv-$C_H3$: where, the scFvs are joined to the $C_H3$ domain, and scFv-SA, SA: streptavidin), compared to full-length mAbs, in various medical applications (Weisser and Hall, 2009).

There are several potential advantages of using scFv fragments over whole antibodies or larger fragments for liposome targeting. These include: i) slower clearance than mAb-targeted liposomes, as Fc-mediated clearance is eliminated (Cheng and Allen, 2008); ii) theoretically lower production cost for scFv fragments generated from bacterial cultures relative to whole antibodies generated from animal ascites or cell culture (Kipriyanov et al., 1997); iii) the ability to select scFv with the desired affinity and specificity using phage display (Pini and Bracci, 2000); iv) the option of engineering tags into scFv constructs, which can aid in their identification and purification (Lindner et al., 1997);

and v) the ability to engineer fully human fragments or fragments with low levels of nonhuman content, which will reduce the risk of immunogenic reactions (Pavlinkova et al., 2001).

In order to achieve coupling of scFv to liposomes, one or more additional cysteine residues are attached to the C-terminus of scFv fragments (Völke et al., 2004). This allows for site-directed conjugation, with the reactive sulfhydryl group(s) located opposite the antigen-binding site. Thus, similar to coupling of Fab' fragments, conjugation of these scFv fragments does not interfere with target cell recognition. ScFv molecules are well established and have been used by several research groups for the generation of targeted liposomes. Expression of scFv fragments in bacteria normally results in a mixture of monomeric and dimeric molecules, the latter being oxidation products of two scFv molecules. Thus, in order to achieve efficient coupling, scFv preparations have to be reduced under mild conditions prior to coupling (Kontermann, 2006).

### 2.13.2. Affibodies as targeting ligands (affisomes)

In recent times, a novel class of small molecules called "affibodies," which can be considered antibody mimics, have been examined for liposome targeting. Affibody molecules are relatively small proteins (6-8kDa) that offer the advantage of being extremely stable, highly soluble, and readily expressed in bacterial systems or produced by peptide synthesis. The binding affinities of affibody molecules are considerably higher compared with the corresponding antibodies (Puri et al., 2009).

The binding pocket of an affibody is composed of 13 amino acids, which can be randomized to bind a variety of targets. In contrast to monoclonal antibody, affibody has following advantages as a targeting ligand. First, the small size of affibody (MW: 6kDa) guarantees its tissue/cell penetration ability. Second, its functional end groups for chemical conjugation are distanced from its binding site. Moreover, affibody has a robust structure, and can be easily synthesized in a large-scale manner (Alexis et al., 2008). All of these advantages make the affibody a valuable ligand for targeted drug delivery and tumor imaging. Wikman et al (Wikman et al., 2004), for the first time, identified an affibody ($His_6$-ZHER2/neu:4) which can specifically bind to the HER2 extracellular domain with a nanomolar affinity (~50 nM). The $His_6$-ZHER2/neu:4 affibody also showed selective binding to native HER2 on breast cancer cells (Wikman et al., 2004). Since then, anti-HER2 affibody has been widely used as an efficient tumor imaging tool after conjugating with radionuclide. Due to the short plasma circulation and fast blood clearance, affibodies are optimal for tumor imaging, but not for the affibody drug conjugates and radiotherapeutics. Therefore, extension of the affibody survival time might be a prerequisite for affibody mediated targeted therapy. The albumin binding technology has been used to extend the plasma half life of affibody. Fusing affibody to the Albumin Binding Domain (ABD), a small protein domain (5kDa), has been shown to elongate the half-life of affibody in mice (Tolmachev et al., 2007)

Recently, anti-HER2 affibody was also employed as a targeting ligand for nano-scaled drug delivery systems (Alexis et al., 2008; Belousova et al., 2008). Alexis et al (Alexis et al., 2008) conjugated the anti-HER2 affibody to poly-(D,L-lactic acid)-poly(ethylene glycol)-maleimide (PLA-PEG-Mal) copolymer, which was used to prepare paclitaxel encapsulated nanoparticles. This nanoparticle formulation was specifically internalized to HER2 positive tumor cells, and subsequently demonstrated cellular toxicity (Belousova et al., 2008). Furthermore, an adenovirus capsid was modified with anti-HER2 affibody to change the natural tropism of the adenovirus vector. The adenovirus fiber was redesigned to include the anti-HER2 affibody without affecting the virion formation. The modified adenoviral vector selectively delivered a dual-function transgene into HER2 positive breast cancer cells (Alexis et al., 2008; Belousova et al., 2008). Recently, Puri et al (Puri et al., 2006) conjugated an 8.3-kDa HER2-specific affibody molecule (Z(HER2:342)-Cys) to the surface of thermosensitive liposomes (called "affisomes") aimed at improving the targeting efficacy of these liposomes for breast cancer treatment. Another study by Beuttler et al (Beuttler et al., 2009) used a bivalent, high-affinity epidermal growth factor receptor (EGFR)-specific affibody molecule (14-kDa) for targeting PEGylated liposomes to EGFR-expressing tumor cell lines. Enhanced cytotoxicity toward EGFR-expressing cells was detected with mitoxantrone loaded affibody targeted liposomes compared to untargeted liposomes in these studies (Beuttler et al., 2009). Since the receptor-binding domains of affibodies may differ from that of antibodies, affisome uptake mechanisms may result in altered outcomes. Therefore, further studies *in vitro* and in animals are needed to establish the projected advantage of affibodies as targeting ligands for liposomes.

**Table 2.4:** Examples of active targeted liposomes under preclinical and clinical trials
Clinical data are obtained from http://www.clinicaltrials.gov

| Targeting ligands/targets | Indications/tumor cells | Status | Reference |
|---|---|---|---|
| Anti-HER-2 MAb/HER2 receptors | BT-474/MCF-7 breast cancer | Preclinical | Kirpotin et al., 2006 |
| Anti-EGFR MAb/EGFR receptor | MDA-MB-468, U87 glioma | Preclinical | Mamot et al., 2005 |
| Anti-MT1-MMP Fab'/MT1-MMP | HT1080 cells | Preclinical | Hatakeyama et al., 2003 |
| Anti-GAH Fab'/GAH | Metastatic stomach cancer | Phase I | Matsumura et al., 2004 |
| Anti-VCAM-1 MAb/VCAM-1 | Human tumor cell line Colo677 | Preclinical | Gosk et al., 2008 |
| NGR peptide/Aminopeptidase N | Orthotopic neuroblastoma | Preclinical | Pastorino et al., 2003 |
| Transferrin/Transferrin receptors | Ovarian cancer cell A2780 | *In vitro* | Krieger et al., 2010 |
| Transferrin/Transferrin receptors | C6 glioma | Preclinical | Ying et al., 2010 |
| Transferrin/Transferrin receptors | Metastatic solid tumor | Phase I | Suzuki et al., 2008 |
| Folate/Folate receptor | Human KB carcinoma | Preclinical | Gabizon et al., 2003 |
| Hyaluronan/CD44 receptor | B16F10 melanoma | *In vitro* | Eliaz et al., 2004 |
| RGD peptide/Integrins ($\alpha_v\beta_3$) | B16 melanoma | Preclinical | Xiong et al., 2005 |
| GPLPLR Peptide /MT1-MMP | Colon 26 NL-17 carcinoma | Preclinical | Kondo et al., 2004 |

**Table 2.5:** Conjugation strategies of active targeted liposomes under preclinical and clinical trials

| Liposome composition | Conjugation strategy used |
|---|---|
| HSPC:Chol (3:2, molar ratio):PEG-PE(0-6 mol %):M-PE(2 mol %) | Anti-HER2 MAb fragments (Fab' or single chain Fv with C-terminus cysteine residue), specific for HER2 expressing breast cancer cells (BT474), were conjugated to liposomes *via* thioether linkage (Kirpotin et al., 2006). |
| DSPC:Chol (3:2, molar ratio):mPEG-DSPE (0.5-5 mol %):Mal-PEG-DSPE | Intact anti EGFR C225 mAb (cetuximab), specific for EGFR- overexpressing MDA-MB-468 tumor cells, was digested with pepsin and the resultant C225-F(ab')$_2$ was reduced with 2-mercaptoethylamine. Thiolated Fab' fragments were covalently conjugated to maleimide groups of liposomes *via* thioether linkage (Mamot et al., 2005). |
| HSPC:Chol (6:4, molar ratio):DSPE-PEG:DSPE-PEG-Mal (9:1, molar ratio) | Intact anti-MT1-MMP mAb (222-1D8), specific for MT1-MMP overexpressing cells (HT1080), was digested first with pepsin and then 222-1D8- F(ab')$_2$ was reduced with cysteamine hydrochloride. Thiolated Fab' fragment were then conjugated to liposomes *via* thioether linkage (Hatakeyama et al., 2003). |
| DPPC:Chol:Maleimidated DPPE (18 : 10 : 0.5, molar ratio) | F(ab')$_2$ fragments of Intact anti -GAH monoclonal antibody, specific for metastatic tumor cancer, were prepared by pepsin digestion. The F(ab)$_2$/Intact GAH was thiolated with 2-iminothiolane and conjugated to liposomes through thioether linkage (Matsumura et al., 2004). |
| SPC:Chol:cyanu-PEG$_{2000}$-PE:DiO (64.5:30:5:0.5, mol%) | Intact anti-VCAM-1 mAb, specific for human non-small cell lung tumor cell line Colo677 which forms solid tumors with VCAM-1 positive vessels, was conjugated to liposomes through chlorine group present in the PE-PEG$_{2000}$-cyanuric chloride *via* nucleophilic substitution (Gosk et al., 2008). |

| | |
|---|---|
| HSPC:CHOL:DSPE-$PEG_{2000}$:DSPE-$PEG_{2000}$-Mal (2:1:0.08:0.02, molar ratio) | NGR peptide which targets aminopeptidase N, a marker of angiogenic endothelial cells (human NB cell lines GI-ME-N, GI-LI-N, HTLA-230, IMR-32, and SH-SY5Y) was used. The additional residues were added to the peptide $NH_2$ terminus to obtain peptides GNGRGGVRSSSRTPSDKYC with a $NH_2$-terminal Cysteine, which were then conjugated to liposomes *via* thioether linkage (Pastorino et al., 2003). |
| EPC:Chol:DSPE-$PEG_{2000}$:DSPE-$PEG_{2000}$-COOH: DSPE-$PEG_{2000}$-$NH_2$ (52:43:4:0.5:0.5, mmol/mmol) | First the liposomes were modified with MAN. In this reaction, the $NH_2$ group of liposomes was coupled with MAN using glutaraldehyde as a coupling agent, as previously reported (Mitra et al., 2005; Ghosh and Bachhawat, 1980). Then the COOH group of liposomes was activated with EDCI and NHS and transferin, specific for brain glioma cell C6, was conjugated to liposomes through amide linkage (Ying et al., 2010). |
| DSPC:Chol:DSPE-PEG (2K):DSPE-PEG(3K)-COOH (2:1:0.16:0.032, molar ratio). | The COOH group of liposomes was first activated with carbodiimide (EDC) and sulfo-NHS. Transferin, specific for transferring receptor overexpressing solid Colon 26 tumor cells, was then conjugated to liposomes through amide linkage (Suzuki et al., 2008). |
| HSPC(55%):Chol(40%):mPEG $_{2000}$-DSPE(4.7%):Folate-$PEG_{3350}$-DSPE | Folate ligand, specific for folate receptor-overexpressing tumors (mouse M109 and human KB carcinomas, and mouse J6456 lymphoma) was used (Gabizon et al., 2003). |
| SPC:Chol:DSPE-PEG:DSPE-PEG-RGD (20:10:1:1, molar ratio) | RGD (Arg-Gly-Asp) peptide, specific for several different integrins ($\alpha_v\beta_3$ and $\alpha_5\beta_1$) expressing murine B16 and human A375 melanoma cells, was conjugated to DSPE-PEG using DSPE-PEG-N-benzotriazole carbonate (BTC). The reaction takes place between α-amines of the RGD and BTC group of the lipid (Xiong et al., 2005). |
| DSPC:Chol: stearoyl-GPLPLR (10:5:1, molar ratio) | GPLPLR peptide, specific for MT1-MMP expressed specifically on the angiogenic endothelium as well as tumor cells (Colon 26 NL-17 carcinoma cells), was used as a ligand (Kondo et al., 2004). |

## 2.14. DOCETAXEL

### 2.14.1 Introduction

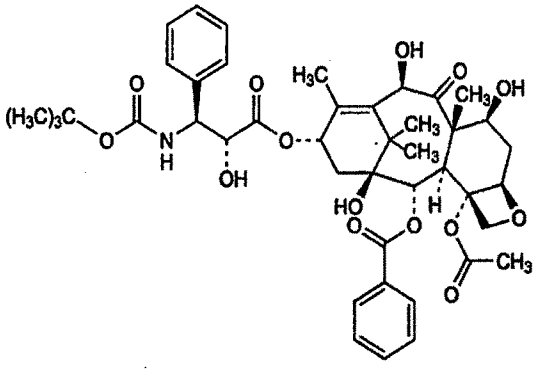
Docetaxel (Taxotere) is an analogue of paclitaxel (Taxol®), obtained by semisynthesis from 10-deacetylbaccatin III, extracted from the needles of the European Yew Tree *Taxus baccata*. Like paclitaxel, docetaxel exerts its cytotoxic properties by inhibiting microtubule depolymerization and promoting tubulin assembly. Docetaxel has shown excellent anti-tumor activity, in both *in vitro* and *in vivo* models, and has generally been found to be more active than paclitaxel. Docetaxel was first administered to cancer patients in 1990 and clinical phase II studies started in 1992.

### 2.14.2. Description

**Chemical name**

The chemical name for docetaxel is (2R,3S)-N-carboxy-3-phenylisoserine,N-*tert*-butyl ester, 13-ester with 5(beta)-20-epoxy-1,2(alpha),4,7(beta),10(beta),13(alpha)-hexahydroxytax-11-en-9-one 4-acetate 2-benzoate, trihydrate.

Docetaxel has the following structural formula:




**Classification:** Antimitotic Agents, anti microtubule Agents.

**Marketed preparations available:** Taxotere, Sanofi Aventis, USA.

### 2.14.3. Physicochemical properties

***Docetaxel***

Empirical: $C_{43}H_{53}NO_{14}$

Molecular weight: 807.9

Appearance and color: A white to almost white powder

***Docetaxel trihydrate***

Empirical: $C_{43}H_{59}NO_{17}$

Appearance and color: A white to almost white powder

Molecular weight: 861.9

Melting point: 168.5° (Liao, Ho et al. 2008)

Solubility: Soluble in ethanol, methanol, chloroform, insoluble in water.

Ultraviolet Spectrum in Aqueous acid (ethanol) - 230, 275, 283nm

### 2.14.4. Physicochemical stability

In acidic media or in the presence of electrophilic agents, opening and/or rearrangement the D ring, as well as in the B ring is observed, depending on the conditions employed. In basic media, cleavage of the ester groups at positions 2, 4 and/or 13 is observed. One of the principal paths of degradation observed, be it in alkaline, neutral or strongly acidic media is the epimerization of the hydroxyl group at position 7 which results in the formation of 7-epi-docetaxel by way of a retro aldol reaction. The degradation of docetaxel can result in products which have reduced activity or are completely inactive. They also demonstrate pharmacological and toxicological profiles completely different from the active principle. The importance of these complex transformations has grave consequences when considering the fact that the pharmaceutical formulations are destined for use in human subjects.

### 2.14.5. Clinical pharmacology

Docetaxel acts by disrupting the microtubular network that is essential for mitotic and interphase cellular functions. It promotes the assembly of tubulin into stable microtubules and inhibits their disassembly, causing inhibition of cell division and eventual cell death. Both docetaxel and paclitaxel bind to the same microtubule site, although the affinity of docetaxel is 1.9-fold higher. Cross-resistance between docetaxel and paclitaxel does not occur consistently. Docetaxel is a radiation-sensitizing agent. It is cell cycle phase-specific (G2/M phase).

### 2.14.6. Pharmacokinetics

**Disposition in the body:** Docetaxel is rapidly distributed throughout the body into body tissue and is extensively metabolised by the hepatic cytochromes of the CYP3A group. Excretion is mainly in faeces (75%) as one major and three minor inactive metabolites and a very low amount of the unchanged drug.

**Protein binding:** > 95%.

**Half-life:** half-lives for $\alpha$, $\beta$ and $\gamma$ phases are 4 min, 36 min, and 11.1 hr, respectively.

**Volume of distribution:** 95 to 150 L/m2 (from various studies), also reported as 113 L.

**Clearance:** 17 to 22 L/h/m2.

**Distribution in blood:** Little interaction with red blood cells.

**Therapeutic concentration:** Four patients with solid tumors, both male and female, were administered with an intravenous dose of 100 mg/m2 docetaxel over 1 to 2 h. A peak plasma concentration of 2.41 mg/L was reached by the end of infusion (Drug-Profile-Clarks 2006).

In another study, 7 patients administered with a 100 mg/m2 dose reached peak plasma concentrations of 3.67 mg/L.

### 2.14.7. Toxicology

Most important dose dependent acute toxicities involved with docetaxel are myelosuppression, peripheral neurotoxicity, moderate immune suppression, febrile

neutropenia, hypersensitivity reactions, fluid retention, nausea, diarrhea, mouth sores and alopecia.

### 2.14.8. Indications and usage

**Breast Cancer:** Taxotere (docetaxel) for Injection Concentrate is indicated for the treatment of patients with locally advanced or metastatic breast cancer after failure of prior chemotherapy.

**Non-Small Cell Lung Cancer:** Taxotere (docetaxel) for Injection Concentrate is indicated for the treatment of patients with locally advanced or metastatic non-small cell lung cancer after failure of prior platinum-based chemotherapy.

### 2.14.9. Dosage and administration

Doses between 55 and 100 mg/m2 body surface are administered for 1 h every 3 weeks; the greater dose is the usual dose. Lower doses are given if adverse reactions are observed during treatment. Patients with hepatic impairment: 75 mg/m2.

### 2.14.10. Analytical methods

Various analytical methods such as HPLC, LC-MS and Radiolabeling methods were reported for the determination of docetaxel.

**Table 2.6. Analytical methods for docetaxel**

| Mobile Phase | Type of sample | Determination | Reference |
|---|---|---|---|
| 1. Acetonitrile: 35 m*M* ammonium acetate buffer (pH 5) : Tetrahydrofuran (45:50:5, v/v). | Plasma | HPLC analysis - UV detection at 227nm | (Ciccolini et al., 2001) |
| 2. Acetonitrile: water (80:20, v/v) containing 0.1% formic acid. | Plasma | LC-MS Detection at 15 v | (Baker et al., 2004) |
| 3. $^{14}C$ labelled docetaxel | Plasma and Tissues | Radioactivity was detected with a Liquid Scintillation Analyzer | (Immordino et al., 2003) |
| 4. Mixture of water and acetonitrile. | Analytical | HPLC analysis - UV detection at 230nm | (Rao et al., 2006) |
| 5. Mixture of acetonitrile: *n*-butylchloride (1:4, v/v). | Plasma | HPLC analysis - UV detection at 230nm | (Loos et al., 1997) |
| 6. Acetonitrile0.02 *M* : ammonium acetate buffer, pH 5, 43:57, v/v | Plasma | HPLC analysis - UV detection at 230nm | (Garg and Ackland, 2000) |
| 7. Methanol: 0.3% phosphoric acid (67:33 v/v) | Plasma | HPLC analysis-UV detection at 229nm | (Ceruti et al., 1999) |

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