Literature review

2. LITERATURE REVIEW

2.1 Cancer and bone metastasis

2.1.1 Participation of Bone Microenvironment in Cancer

Bone is one of the most favored sites of solid tumor metastasis, indicating that the bone microenvironment provides a fertile soil for the growth of many human tumors. In fact, patients with the most common solid tumors, such as breast, lung, and prostate carcinomas, may have the major portion of the tumor burden present in bone at death. Typical clinical diagnosis in bone metastasis include pain, spinal cord compression, and pathologic fractures (Rubens, 1998). Pain is usually the first symptom and results from mechanical or chemical stimulation of pain receptors in the periosteum/ endosteum by the growing tumor mass. Spinal cord compression results from expanding extradural tumor growth, spinal angulation secondary to vertebral collapse, or dislocation of the vertebra after pathologic fracture. Back pain, motor weakness, sensory loss, and autonomic dysfunction are common symptoms of spinal cord compression. Pathologic fractures occur as a result of the tumor mass weakening the bone and are associated with both osteolytic and osteoblastic bone lesions (Rubens, 1998; Orr et al, 2002).

Bone metastases can be categorized into three distinct phenotypes: osteolytic, osteoblastic, and mixed lesions containing elements of both (Roodman, 2004). Patients with advanced bone metastases from primary breast cancer or prostate cancer eventually develop a mixture of both osteoblastic and osteoclastic lesions to provide cancer cells a better environment for survival. For example, in breast cancer bone metastasis, majority of bone lesions in patients are osteolytic, whereas approximately 15% to 30% are osteoblastic reactions. Similarly, prostate cancer shows higher osteoblastic reaction with some degrees of osteolytic reaction in bone metastasis patients (Roodman, 2004; Yi, 2002). These phenotypes reflect the perturbation of normal bone remodeling processes by the presence of tumor cells. This suggests that the pathology of each type of lesion is not static; rather, the observed phenotype in each metastatic lesion results from a shift in the dynamic equilibrium of normal bone remodeling.

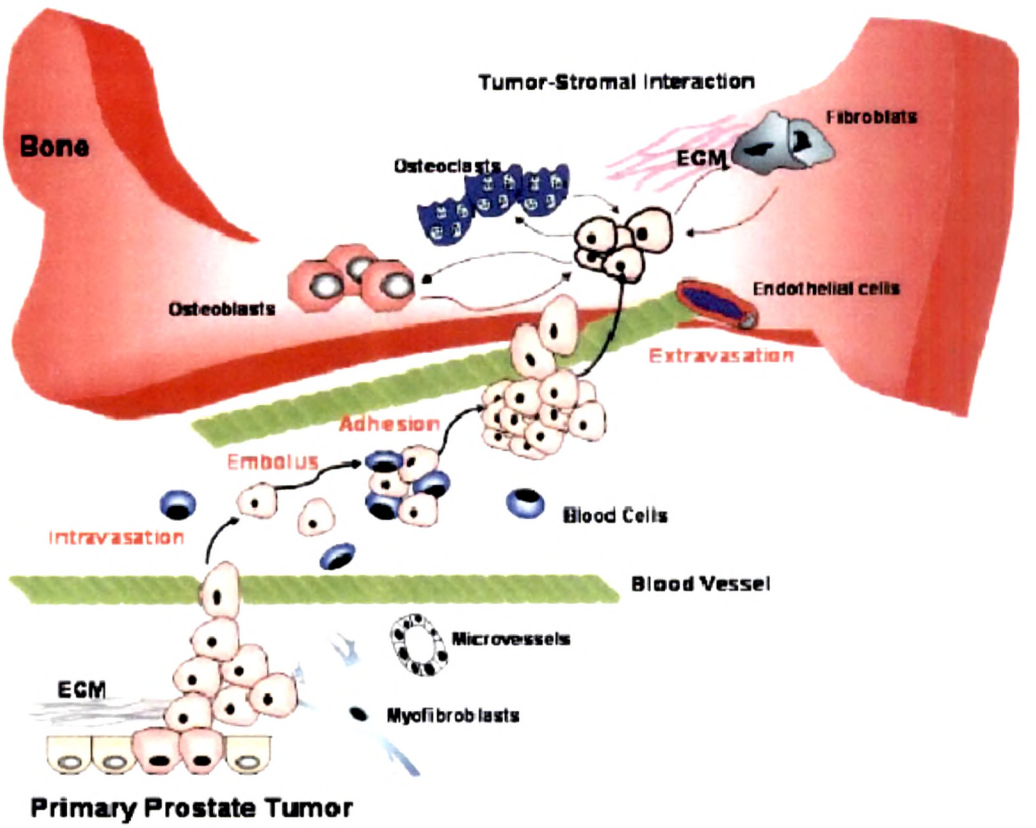


Figure 2.1: Bone microenvironement, tumor cell inoculation and colonization

The progression of prostate cancer from the androgen-dependent to androgen-independent and bone metastatic state is considered a poor and generally a more rapidly lethal prognosis. To understand the molecular basis of disease progression and develop rational new therapeutic approaches for targeting prostate cancer bone metastasis, the multi-step processes that lead to prostate cancer metastasis of bone, needs to be identified and understood. As depicted in Fig 1, at the site of primary cell growth, prostate cancer cells are expected to interact with prostate stromal cells and gain the ability to extravasate into the bloodstream. In the blood, prostate cancer cells are expected to survive and move as an embolus before adhering to bone marrow-associated endothelial cells. The attachment and interaction of prostate cancer cells to marrow endothelial ECMs could activate the invasive properties of prostate cancer cells and allow their extravasation into the marrow space. At the final step of this progression, prostate cancer cells interact directly with bone stromal fibroblasts, osteoblasts and osteoclasts through a series of

soluble factors (ex, RANKL) via cell surface receptor (ex, RANK) to survive, proliferate, migrate, invade and eventually replace the bone marrow components. Recent studies of cancer metastasis demonstrated that chemokines released by bone stromal cells induce cancer cells migration to the bone area, such as the CXCL12- CCR4 interaction in breast cancer bone metastasis models (Prasad et al, 2004). It also merges the idea that self-seeding of cancer cells in bone induces the colonization of cancer cells in the distant region (Norton and Massague, 2006). In the seed and soil; and self-seeding hypotheses, the first group of cancer cells migrates to many distant organs and later either die out or leave due to immune surveillance or environmental restrictions; however, those cells that do manage to interact with the target organ, such as bone, could introduce permanent changes in those microenvironments to serve as "fertilized" soil and also release chemokines leading cancer cells to such good environments at later stages of cancer development. Those cancer cells that do survive the process would find themselves in a more welcoming microenvironment which the pioneer cancer cells contributed to. This coevolution of cancer cells and stroma that creates such microenvironments may be reflected by the presence of tumor and stroma-specific gene expression patterns (Cooper et al, 2003, Romanenko et al, 2007). To understand the cellular and molecular basis of the prostate cancer bone–stroma interaction, it is essential to delineate how the soluble growth factors and extracellular matrices participate reciprocally in the progression of prostate cancer towards androgen independence and bone metastasis.

2.1.2 Vicious cycle of cancer and bone stroma

Human prostate cancer is predominantly osteoblastic, the established human prostate cancer cell lines inoculated and grown in the bone of immune compromised mice yield both osteoblastic and osteolytic lesions. Apparently, prostate cancer cells can participate in the process of bone turnover by exhibiting properties similar to osteoblasts. Much evidence supports this interesting phenotype of prostate cancer cells, in which they behave like osteoblasts. Prostate cancer cells express both soluble and membrane-bound RANK ligands, and shown to participate directly in osteoclastogenesis (Keller and Brown, 2004).

Since bone-homing prostate cancer cells seek to adhere, colonize, and survive in bone, it is of pivotal importance to find out how prostate tumor and bone cells interact. One attractive hypothesis is that prostate cancer cells may behave like osteoblasts and functionally participate in bone turnover. By markedly increasing the basal rate of bone turnover, they may further enhance prostate cancer cell colonization in bone (Schneider et al, 2005). This hypothesis is supported by some clinical observations where bisphosphonates, an effective class of agents that slow down or inhibit bone resorption, have been shown to reduce cancer cell colonization in experimental models of prostate and breast cancers (Berruti et al, 2001). In men harboring prostate cancer, there is evidence that increased bone resorption occurs on castration. Whether these changes in bone turnover subsequent to hormonal manipulation or bisphosphonate treatment after prostate cancer cell colonization in bone affect the natural history of prostate cancer progression should be the subject of future thorough investigation.

2.1.3 Factors influencing bone metastasis

Chirgwin (2000) and Mundy (2002) presented the concept of a vicious cycle involving TGFβ produced by bone cells that promotes the production of PTHrP by tumor cells, which in turn stimulates bone turnover by enhancing osteolytic reaction in the bone. Increased release of TGFβ could result from rapid bone turnover and this triggers more PTHrP production by cancer cells. The production of PTHrP by tumor cells will induce osteolytic cells to express an increased level of RANK ligands.

The increased RANK ligands will help promote osteoclast formation/activation and subsequently increase bone resorption. The enhanced resorptive process by osteoblasts and osteoclasts leads to 'bone pitting' and subsequent colonization by breast cancer cells in the skeleton and associated bone destruction often observed in breast cancer patients. It is likely that the similar proposed 'vicious cycle' between TGFβ, PTHrP, RANK ligands and osteolytic breast cancer cells could also occur in prostate cancer. Interrupting the vicious cycle in breast cancer models using anti-PTHrP antibodies or osteoprogerin (OPG) has shown to reduce colonization of breast cancer metastasis to bone and prevent prostate cancer growth in the skeleton (Saito et al, 2005; Zhang et al, 2001)

Patients with advanced prostate cancer bone metastases often have androgen-independent prostate cancer (AIPC) and are hormone refractory, hence bone-associated growth factors and cytokines preferentially stimulate the growth of hormonally independent prostate cancer cells. Several factors are regulated by prostate cancer in the bone, such as bFGF, IGF, PDGF, EGF, and TGFβ (Lee et al, 2003) which can reciprocally regulate prostate cancer invasion and survival in bone microenvironment. However, some bone growth factors, such as TGFβ, can inhibit or stimulate the growth of prostate cancer cells, depending on their phenotype (Orr et al, 2000). Other factors, such as CXCL12, may synergize the mitogenic effect of other growth factors, which suggests CXCL12 and its receptor (CXCR4) may play a role as prostate cancer bone metastasis homing signals (Taichman et al, 2002). The level of CXCR4 increases with the malignancy of prostate cancer cell lines by both RT-PCR and Western blot analysis, and increased expression of CXCR4 also increased spreading to bone in animal studies. An *in vitro* study of cellular spreading in basement membrane indicated that spreading can be inhibited by CXCR4 antibody. These findings suggested that chemokine and its receptor could also play an important role in prostate cancer bone metastasis.

2.1.4 Systematic spread of the tumour cells

The development of bone metastasis is a multi-step process consisting of the following sequence of events:

- ✓ Tumour growth, detachment of cancer cells, and invasion of the prostate stroma;
- ✓ Neoangiogenesis;
- ✓ Escape from the prostate by intravasation;
- ✓ Survival in the circulation;
- ✓ Chemoattraction and arrest (docking and locking) in the bone marrow endothelial vessel wall;
- ✓ Extravasation; and

- ✓ Establishment of the metastatic microenvironment (osteoblastic metastasis) via the cross-talk between prostate cancer and bone cells.(Koutsilieris, 1993; Koutsilieris, 1995)

During the early stages of the cancerous transformation (carcinoma in situ), small clusters of dysplastic cells remain confined in the otherwise normal prostate gland. In the course of time, these malignant prostatic epithelial cells evolve and spread to the surrounding prostate stroma, forming an invasive tumour. The localized tumour eventually becomes more aggressive, expands and begins to penetrate into nearby structures such as the seminal vesicles and the bladder. Nutrients are initially supplied by simple diffusion, but the increased demand of the expanding tumour mass is further supplied by an extensive neovasculature which is established via the synthesis and secretion of angiogenic factors (Koutsilieris, 1993). This extensive vessel network increases the chance of tumour cells to reach the bloodstream and cause metastasis. In order to enter the lymphatic and systemic circulation, the malignant cells will have to detach from adjacent cells, dramatically increase their motility, attach to and disrupt the endothelial basement membrane, and finally migrate into the tumour blood vessels (Koutsilieris, 1995). During these sequences of metastatic progression, tumour cells will undergo changes in their proliferative, survival and invasive abilities, while their number is greatly reduced through cell clone selection of those comparatively few cells that can successfully survive and grow in the distal sites. Detachment of normal epithelial cells from the extracellular matrix (ECM) results in FAS-induced apoptosis, a phenomenon called 'anoikis'. Therefore, the invading tumour cells must develop resistance to anoikis in order to continue their migration and aberrant growth (Rennebeck et al, 2005)

Following their intravasation, the metastatic prostate cancer cells must survive through a number of hostile circulatory compartments, including the blood and lymphatic channels. Approximately 85% of the tumour cells that intravasate to the circulation will undergo a rapid phase (<5 minutes) of intravascular cell death. Most of the circulatory prostate cancer cells will either die in the blood vessels or rapidly after extravasation. This was recently confirmed using luciferase-labelled PC-3 human prostate cancer cells that were

administered to nude mice by intracardiac injection. No viable cells could be detected at 24 hours post-injection, indicating that most cells either died or were metabolically inactivated (Rosol et al, 2003). The two main causative mechanisms of tumour cell death in the circulation are the mechanical stress of vascular transportation and the host immune defence.

Prostate cancer cells that circulate as part of a fibrin clot surrounded by other tumour cells and platelets can gain a survival advantage because such aggregates may protect the cells from the mechanical shear forces and facilitate their arrest in the target organ microvasculature (Chay et al, 2002). Furthermore, prostate cancer cells evade the immune system by down-regulating their expression of the major histocompatibility complex (MHC) class I. Indeed, shedding of MHC class I chain-related molecules has been positively correlated with the severity of prostate cancer disease as well as with natural killer (NK) cell impairment (Wu et al, 2004). In addition, complete loss of MHC class I expression has been reported in 34% of primary prostate tumour cells and in 80% of prostate cells derived from lymph-node metastases (Blades et al, 1995).

Prostate cancer metastasis occurs according to a predictable pattern. The tumour cells prefer to infiltrate the trabecular bone of the axial skeleton (i.e. the lumbar spine, ribs, pelvis) as well as the proximal ends of the femur. Despite its clinical and physiological significance, the cause of this preference still remains to be fully elucidated. Two theories which are not necessarily mutually exclusive have been historically proposed to explain why prostate cancer cells tend to colonize the bone. The first theory is based on the existence of a network of veins, called the Batson plexus, that drain the lower vertebral column pelvic girdle which receives blood from the prostate (Batson, 1967). A tight interconnection exists between the Batson plexus and the marrow spaces of the vertebral column, where a large number of metastatic prostate tumour cells are found. The routine detection of tumour cells in the circulation of prostate cancer patients supports the notion of haematogenous spread (Ghossein et al, 1995).

Entry into the bone marrow is facilitated by the arrest of circulating prostate cancer cells in the vascular beds which form sinusoids characterized by their large diameter and reduced blood flow rate. Furthermore, prostate cancer cells attach to the bone endothelium more efficiently compared to the endothelia of other organs via the recognition of certain protein receptors (ex. selectins, integrins and cadherins) and demonstrate an inherent tropism to the bone microenvironment where they establish a particularly hospitable niche (Lehr and Pienta, 1998). In order to reach the bone surface, tumour cells must first 'dock' (low-affinity binding) on a bone-marrow endothelium-specific lectin and then 'lock' (firmer cell adhesion) on a bone-marrow endothelium-specific integrin ('docking and locking' hypothesis) (Honn KV & Tang, 1992). Following this binding to the bone-marrow endothelium, the tumour cells must produce proteolytic enzymes in order to disrupt and traverse the basement membrane of the bone-marrow microvessels and finally extravasate into the bone microenvironment (Koutsilieris, 1995).

2.1.5 Colonizing the bone

The osseous tissue is composed of two biologically distinct structures: the dense, mineralized cortical or compact bone (approximately 85% of the total bone mass) and the spongy, more metabolically active cancellous or trabecular bone (approximately 15% of the total bone mass) which makes up the bulk of the interior of most bones, including the vertebral bodies and pelvis. The external layer of the trabecular bone contains the multicellular red bone marrow, where the production of osteoblasts and osteoclasts takes place by differentiation of stromal and pre-osteoclast haematopoietic cells, respectively. The most frequent sites of skeletal metastasis are the more metabolically active bones with high content of red bone marrow (Bogdanos et al, 2003). The reticulate vasculature of the bone marrow provides a rich supply of oxygen, nutrients and growth/survival factors that support prostate cancer cell survival and proliferation. During their initial arrival in the bone marrow, prostate cancer cells should theoretically be equally distributed into the metabolically active bones (Bogdanos et al, 2003). A crucial first step during the initial migration stage is the activation of local bone resorption which debulks the bone, thus permitting the seeding of cancer cells, and liberates survival and growth

factors that promotes tumour progression. This initial osteolytic phase is orchestrated by the prostate cancer cells and performed by osteoclasts (Koutsilieris, 2006). So far, direct tumour-cell-mediated bone resorption has not been convincingly demonstrated in the absence of osteoclasts.

Tumour cells achieve local bone resorption by chemotactically attracting osteoclast precursor cells (pre-osteoclasts) of the monocyte/macrophage cell line and stimulating their fusion and formation of mature osteoclasts. This osteoclastogenesis process is regulated by the nuclear factor kB (NFkB) ligand (RANKL)/RANK/osteoprotegerin (OPG) system (Figure 2.1). RANKL is mainly expressed on the surface of osteoblasts, whereas its specific receptor (RANK) is expressed on osteoclast precursors. Stimulation of RANK by its ligand induces osteoclast formation and activation (Hsu et al, 1999). The soluble glycoprotein OPG is a decoy receptor that binds to RANKL and thus inhibits RANKL-RANK interaction. OPG also serves as a survival factor for prostate cancer cells because it can bind to tumour-necrosis-factor-γ (TNF- γ) related apoptosis-inducing ligand (TRAIL) and thereby inhibit TRAIL-induced cancer-cell apoptosis. Nevertheless, OPG administration significantly reduces prostate cancer progression in bones because it inhibits tumour cell migration and bone resorption (Mori et al, 2007).

Prostate cancer cells have been shown to express several other humoral factors that regulate osteoclastogenesis, including the macrophage colony-stimulating factor (M-CSF), members of the transforming growth factor b (TGF-b) superfamily, parathyroid-hormone-related protein (PTHrP), urokinase-type plasminogen activator (uPAplasmin) mediated activation of the matrix metalloproteinases (MMPs; specifically MMP-2 and MMP-9) as well as interleukin-1 (IL-1) and interleukin-6 (IL-6) (Fizazi et al, 2003). The osteoclast-mediated bone resorption gives tumour cells the opportunity to penetrate into the bone matrix and alter the local cytokine milieu in favour of bone formation, thereby producing osteoblastic lesions. Therefore, in contrast to other metastatic tumours (e.g. breast and lung cancers) that are characterized by an ongoing osteolytic vicious cycle, prostate cancer bone metastasis initially displays osteolytic activity that eventually transitions to an osteoblastic predominance (Koutsilieris, 1993). This explains why

systemic indices for both osteoblastic (increased osteocalsin and bone-specific alkaline phosphatase) and osteolytic activity (increased cross-linked N-telopeptide of type-I collagen and deoxypyridinoline) are detected in patients with skeletal metastasis of prostate cancer.

2.1.6 Sheltering inside the bone marrow

The bone matrix and its cellular constituents (i.e. osteoblast-like cells; osteocytes and lining cells) are major direct or indirect sources of growth factors such as insulin-like growth factor 1 (IGF-1), TGF-b1, bone morphogenic proteins (BMPs), basic fibroblast growth factor (bFGF), IL-6, endothelin-1 (ET-1) and PTHrP that are also produced by metastatic prostate cancer cells and act as survival factors on prostate cancer cells (Koutsilieris, 1986). These mitogens are both autocrine/paracrine effectors of tumour cell survival and modulators of bone remodelling via the stimulation of osteoblast activity which leads to deposition of new bone and the increase of OPG expression by the prostate cancer cells, which in turns reduces local osteoclastogenesis and osteoclast survival (Mitsiades et al, 2004). This osteoblastic production and deposition of woven bone (composed of randomly oriented, loosely packed collagen strands that form bone with suboptimal strength) is regulated by the core binding factor-1 (Cbfa1 or Runx-2), an osteoblast-specific transcription factor that controls the expression of significant components of the bone extracellular matrix such as osteocalcin, bone sialoprotein (BSP), osteopontin and type-I collagen. In addition to producing proosteoblastic factors, prostate cancer cells functionally participate in the process of bone matrix formation by gaining an osteoblast-like phenotype (osteomimicry) (Tenta et al, 2005).

The increased local bioavailability of survival factors, mainly IGF-1, in the microenvironment of bone metastases readily supports the survival and growth of prostate cancer cells. Increase in local IGF-1 is caused by elevated concentrations of urokinase type plasminogen activator (uPA), which is synthesized by metastatic prostate cancer cells as a single-chain precursor which is converted to active uPA by human kallikrein 2 (hK2) or plasmin but not by PSA (human kallikrein) (Koutsilieris, 1993). The secreted uPA then binds to its receptor (uPA-R) on the surface of osteoblasts, activating

proteolytic activity at sites adjacent to the osteoblasts and leading to local increase of proteolysis, due to either direct protease activity of uPA or indirect uPA-mediated generation of plasmin and subsequent activation of MMPs (Koutsilieris and Polychronakos, 1992). IGF-binding proteins (IGFBPs) are major targets of uPA-mediated proteolysis. The IGFBPs exhibit higher affinity for IGFs than the type-I IGF receptor (IGF-R) and therefore regulate extracellular IGF bioavailability. Consequently, the uPA-mediated hydrolysis of IGFBPs (mainly IGFBP-3) releases active IGF-1, significantly increasing its local bioavailability which, in turn, leads to IGF-R-mediated proliferation of osteoblasts and prostate cancer cells (Koutsilieris and Polychronakos, 1992). It is therefore conceivable that IGFBP-3 hydrolysis optimizes tumour cell survival at the sites of bone metastasis. Moreover, uPA is known to activate latent-TGFb1 which is produced by prostate cancer cells and osteoblasts; and also to exert important regulatory functions in prostate cancer bone metastasis (Reyes-Moreno et al, 1995). Evidently, the convergent outcome of the uPA/plasmin/IGF axis is to stimulate proliferation of prostate cancer cells and induce the osteoblastic reaction (Mitsiades and Koutsilieris, 2001).

2.2 Targeting molecule platforms

2.2.1 Antibodies or antibody fragments

Antibodies were the first macromolecular ligands used for targeted delivery (Maynard and Georgiou, 2000). The use of monoclonal antibodies (mAb) became widespread after the discovery of hybridoma technology. The selection process can be designed to improve the properties of the ligand such as stability, affinity, selectivity and internalization. Recent advances in protein engineering have led to the development of single chain antibodies, antibody fragments (Fab or scFv) and dibodies (Weiner and Adams, 2000). Antibody fragments show less immune response and can be stabilized with disulfide bond or chemically crosslinked. There are several examples of FDA approved antibodies in clinical practice today, including Rituxan - a chimeric anti-CD20 antibody effective against CD20 B-cell non-Hodgkin's lymphomas (Nowakowski and Witzig, 2006). Other examples include anti- HER2 Herceptin (Piccart et al, 2005), anti-EGFR Erbitux (Reck et al, 2005), and anti-VEGF Avastin (Willett wt al, 2004).

Recent investigations have focused on exploiting the affinity of these antibodies for their respective antigens to 'arm' them with chemotherapy drugs such as doxorubicin (Inoh et al, 2006), methotrexate (Vitols et al, 1995), and calicheamicin (Sievers et al, 2001) and radionucleotides (Sharkey and Goldenberg, 2006). Despite much efforts, Mylotarg® (Sievers et al, 2001) is the only FDA approved antibody-toxin conjugate in practice today. The payload and the chemical conjugation of the drug to antibodies remain the major limitations of chemoimmunoconjugates. Targeted drug delivery carriers are being functionalized with antibodies or antibody fragments to overcome some of these limitations (Nielsen et al, 2002). Furthermore, the conjugation of multiple antibodies to each nanocarrier enhances their avidity, and nanocarriers can be surface functionalized with multiple distinct antibodies to overcome tumor heterogeneity. Long-circulating doxorubicin encapsulated immunoliposomes conjugated to human HER2 antibodies have been developed and evaluated in vivo (Kirpotin et al, 2006). After intravenous administration, nontargeted and targeted liposomes similarly accumulated in the tumors; however, targeted liposomes had a significantly higher anti-tumor efficacy, presumably due to the HER2 mediated internalization of targeted liposomes and intracellular drug release vs. the nontargeted liposomes that released the drug locally into the extracellular space (Kirpotin et al, 2006).

2.2.2 Peptides

It is now well accepted that the binding affinity, stability, and the size of the ligand play a critical role for successful targeting. Peptides are small, synthetic molecules that can be manufactured in large quantities with excellent quality control. Peptides are more stable than antibodies and unlikely to be immunogenic. The discovery of new peptide targeting domains has been successful due to the development of peptide library screening methods (e.g., phage display) (Brissette et al, 2006). Peptides that contain RGD (Arg- Gly-Asp) domains can preferentially bind cells in tumor microvasculature that express integrin (Pasqualini et al, 1997). This strategy has been used experimentally to deliver chemotherapy and radioactive molecules selectively to tumors (Bibby et al, 2005). Moreover, cyclic RGD peptide (EMD 121974 or Cilengitide is now under phase II

clinical evaluation for the treatment of several tumors including androgen independent prostate cancer and recurrent *Glioblastoma multiforme* (Beekman et al, 2006).

Other peptide-based targeting technologies have been developed that provide several new platforms for generating target specificity. Using the A-domain of various native human receptors as a platform for modification, single chain proteins that can bind to multiple regions of target molecules using multiple domains have been developed and named avidity multimers or avimers (Silverman et al, 2005). In a proof of concept study, an avimer specific for IL-6 (an acute phase inflammatory mediator) was demonstrated to be non-immunogenic in mice, as well as an inhibitor of IL-6 function in vivo. More generally, this highlights the potential of this class of ligand to antagonize proteins, which may ultimately find application in cancer therapy. Functional, single-domain heavy chain antibodies, also known as nanobodies, have been raised against cancer targets, which either antagonize receptor function or deliver an enzyme for prodrug activation both for therapeutic benefit (Cortez et al, 2004). Yet another important strategy employing phage display technology used a 58 residue helical domain of staphylococcal protein A as a platform to develop polypeptide targeting ligands named affibodies (Hansson et al, 1999). Affibodies against a variety of cancer-related targets have been developed and are now commercially available, including: EGFR, HER2, and transferrin.

2.2.3 Small molecule targeting

In general, small molecules are very attractive as targeting ligands due to their low cost and ease to conjugate with drugs such as imaging probes (quantum dots, etc.) and nanoparticles (Gabizon et al, 2004). The small size of the targeting ligand allows the functionalization of multiple molecules on single nanoparticles. In this regard, folic acid and sugar molecules have been extensively used. Vitamin folic acid which is required for cell survival and folate receptors is homogeneously over-expressed in many cancer cells (Ross et al, 1994). Folic acid receptors (FR) interact with high affinity folic acid conjugates and transport the conjugates via receptor mediated endocytosis, before it recycles back to the cell surface (Kamen and Capdevila, 1986). Thus, it is not surprising that folate targeted nanoparticles have shown to be effective in a number of tumors using

liposomes or polyplexed nanosystems (Park et al, 2005; Quintana et al, 2002). However, immunochemistry studies have shown the overexpression of folate receptors in normal tissues like placenta and kidneys. This raises some issues for possible clinical translation of folate bioconjugates. In addition, folate-targeted stealth nanoparticles need to be carefully designed to avoid possible steric hindrance of free PEG chains. Ideally, the folate ligand should be accessible to enhance the specific binding of folate bioconjugates (Gabizon et al, 1999).

Cell surface lectins have been shown to be overexpressed on numerous cancer cells and to internalize glycoconjugates via a receptor mediated endocytosis mechanism. The presence of cell surface membrane lectins, which recognize specific sugar molecules (lactose, galactose, mannose) represents an interesting approach for targeted delivery or imaging (Ohannesian et al, 1995). However, to compensate for the weak binding affinity of carbohydrates, multiple or multivalent molecules should be conjugated to the nanocarrier. Targeting efficacy of galactosylated macromolecular carriers were shown to depend on galactose ligand concentration (Managit et al, 2003). In addition, the conjugation of galactoside residues with N-(2-hydroxypropyl) methacrylamide copolymers (HPMA) resulted in a significant increase in three different colon cancer cells (David et al, 2002). Surprisingly, the trivalent galactoside bioconjugates did not show higher binding affinity.

Weissleder et al. have demonstrated that small molecules can change the biological affinity of nanoparticles used for imaging specific tissues. Using a high-throughput screening based on surface chemistry, 146 different small molecules were conjugated to nanoparticles and the results showed that very small molecules can drastically affect the binding of nanoparticles to different cell lines and pancreatic tumor xenografts (Weissleder et al, 2005).

2.2.4 Aptamers

Nucleic acid aptamers are single stranded DNA, RNA or unnatural oligonucleotides that fold into unique structures capable of binding to specific targets with high affinity and specificity (Nimjee et al, 2005). Nucleic acid aptamers are a novel class of ligands that

are small (10–20 kDa), non-immunogenic, easy to isolate, and exhibit high specificity and affinity for their target antigen, at best in the picomolar scale (Farokhzad et al, 2006). In addition, aptamers are synthetic molecules that can be easily modified and scaled up for synthesis. The utility of aptamers for possible clinical translation has been possible due to chemical modification allowing a higher stability. Systemic evolution of ligands by exponential enrichment (SELEX) (1015 different sequences) has shown to be very powerful for the selection of high affinity targeted ligands, and multiple selection protocols had been reviewed (Gopinath, 2007). The unique antigen-aptamer binding mechanism had led to the isolation of ligands with high binding affinity. It was shown that aptamer-antigen interaction occurs by a complex mechanism involving the folding of the aptamer on its antigen like a 'paper clip'(Hermann and Patel, 2000). In short time since Szostak and coworkers (1998) and Tuerk and Gold (1990) independently described the ground-breaking methodology for in vitro evolution of aptamers, this class of ligands had emerged as an important arsenal in research and clinical medicine.

Recently, the first proof-of-concept for drug delivery vehicles utilizing aptamers as targeted delivery of drug encapsulated nanoparticles (Farokhzad et al, 2004), and subsequently showed the efficacy of similarly designed nanoparticles against prostate cancer tumors in vivo (Farokhzad et al, 2006). The efficacy study showed that a single intratumoral injection of aptamer targeted nanoparticles loaded with docetaxel allowed all the treated mice to survive more than three months in contrast to other controls. More recently, a novel strategy for targeted doxorubicin delivery to cancer cells using an aptamer-Dox physical conjugate was successfully evaluated (Bagalkot et al, 2006). Other strategies involving aptamers in cancer therapy are targeting the growth factors PDGF (Zhou et al, 2006) and VEGF (Kim et al, 2002) for delivery of therapeutic or diagnostic agents, discovering new cancer-associated antigens using cell-SELEX (Shangguan et al, 2006), and delivering toxins (Chu et al, 2006) or siRNA (McNamara et al, 2006).

2.3 Novel Targets for cancer

2.3.1 Vascular endothelial growth factor receptor

Vascular endothelial growth factor receptor (VEGFR) is considered the most relevant inducer of tumor angiogenesis for its induction of the VEGF receptor signaling cascade. Tumor hypoxia and oncogenes upregulate VEGF levels in the neoplastic cells, resulting in an upregulation of VEGF receptor-1 (also known as fms-like tyrosine kinase) and VEGF receptor-2 (fetal liver kinase-1, flk-1) on tumor endothelial cells (Kremer et al, 1997). Expression of VEGF corresponds to the degree of vascularization, as seen by in situ hybridization and immunohistochemistry (Brown et al, 1993).

The approach to targeting VEGFR-2 has been investigated for various nanoparticulate systems including delivery of boronated dendrimers and radioisotype-labeled polymerized liposomes. For the delivery of boronated dendrimers to the neovasculature, the human recombinant VEGF121 molecule has been conjugated to the surface of the dendrimers (Backer et al, 2005). Using radioisotype-labeled polymerized liposomes, VEGFR-2 receptors were targeted by anti-VEGFR-2 mAb-labeled liposomes radiolabeled with 90Y (anti-VEGFR-2 mAb-NP-90Y). The anti-VEGFR-2 mAb-NP-90Y treatment (0.36 μg/g anti-VEGFR-2 mAb, 5 μCi/g 90Y) was more effective in tumor growth delay than anti-VEGF-2 mAb alone or conventional radioimmunotherapy with 90Y-labeled anti-VEGFR-2 MAb in the mouse melanoma model, K1735-M2 tumor. The anti-VEGFR-2 mAb-NP-90Y was better at limiting tumor growth compared to an isotype-matched control antibody (IC-Ab and IC-Ab-NP-90Y). Staining against anti-CD31, a differentiation molecule expressed on vascular tumors, showed a decreased vessel density in tumors treated with anti-VEGFR-2 MAb-90Y, but not in tumors treated with IC-Ab-NP-90Y (Li et al, 2005). These examples reveal that targeting VEGFR-2 can allow successful reduction of vessel density in tumors.

The method for targeting VEGF has been found to be effective in down regulating VEGFR-2 for a decrease in angiogenesis. This method was used with dextran magnetic nanoparticles (DMNs) conjugated to radiolabeled 131I-anti-VEGF mAbs (Sc-7269) for targeting of VEGF secreted by hepatic carcinoma cells in nude mice bearing human liver cancer (Chen et al, 2006). Four treatments were examined 131I control, 131I-Sc-7269 radiolabeled antibody, 131I-labeled Dextran Magnetic Nanoparticle (DMN), and 131I-

Sc-7269-DMN radiolabeled actively targeting nanoparticle. The 131I-Sc-7269-DMN showed statistically significant differences for tumor growth delay and tumor inhibition rates compared to the other treatments.

2.3.2 αvβ3 Integrin

The targeting scheme for the αvβ3 integrin has centered upon the three amino acid sequence Arginine-Glycine-Aspartic Acid (RGD). The αvβ3 integrin is an endothelial cell receptor for extracellular matrix (ECM) proteins harboring the RGD sequence, which includes von Willenbrand factor, fibrinogen (fibrin), vitronectin, thrombospondin, osteopontin, and fibronectin (Li et al, 2005). The αvβ3 integrin is highly expressed on neovascular endothelial cells and is important in the calcium-dependent signaling pathway leading to endothelial cell migration (Nisato et al, 2003). Endothelial cells undergoing angiogenesis experience at least three cellular alterations, including an increase in proliferation, increase in locomotion, and endothelial cell interaction with the ECM. These alterations are directly related to the adhesion processes of the αvβ3 integrin (Nisato et al, 2003). The most prevalent method for targeting the αvβ3 integrin is RGD sequences harbored on peptides and nonpeptide mimetics. Hood et al. emphasized αvβ3 targeting by an RGD non-peptide mimetic coupled to a nanoparticle for anti-angiogenesis therapies (Hood et al, 2002). The targeted nanoparticles were coupled to cDNA encoding ATPμ-Raf tagged with the FLAG epitope. These nanoparticles were proven to cause tumor regression in M21-L melanomas, where apoptosis staining, TUNEL, showed concentric rings of apoptosis among tumors cells proximal to each apoptotic vessel.

A cyclic RGD pentapeptide was conjugated to the surface of doxorubicin-loaded micelles at different densities: 5, 16, and 76% of all PEG chains in the micelle. It was found through flowcytometry and confocal microscopy that a higher density of RGD sequences led to a higher level of cellular internalization of the micelles over the range of RGD densities. A 30-fold enhancement in micelle internalization was achieved with 76% RGD-functionalized doxorubicin-loaded micelles as compared to the non-targeted micelles (Nasongkla et al, 2004).

An important characteristic of the αvβ3 integrin is that it is intrinsically associated with VEGFR-2 signaling. Upon αvβ3 integrin binding to the components that harbor the RGD sequence, there is an upregulation of VEGF signaling in cell cultures. By blocking αvβ3 integrin binding, there would be a reduction in VEGF signaling, proving the use of αvβ3 blocking agents for anti-angiogenesis (Ruoslahti, 2002).

2.3.3 Vascular cell adhesion molecule

Vascular cell adhesion molecule-1 (VCAM-1), an immunoglobulin like transmembrane glycoprotein, is an optimal target as it is virtually absent on normal human vasculature, yet readily inducible by angiogenesis. During tumor migration and angiogenesis, integrins bind to ECM proteins or cell surface immunoglobulin proteins (i.e. vascular cell adhesion molecule-1 (VCAM-1)) [71]. VCAM-1 is expressed on the surface of endothelial cells during inflammation and cancer and promotes cell-to-cell adhesion (Ruoslahti et al, 1999). In cancer, increased VCAM-1 expression is linked to lymphomas and leukemias, which include Hodgkin's disease, B-cell lymphocytic leukemia, lung and breast cancer, melanomas, renal cell carcinoma, gastric cancer, and nephroblastoma (Dienst et al, 2005).

The first in vivo study of VCAM-1 targeted immunoliposomes performed by Gosk et al. showed extensive colocalization in the endothelial cells of Colo 677 xenograft tumors in mice. Immunoliposomes conjugated to anti-VCAM-1 monoclonal antibody (mAb) (M/K-271) showed a non-significant increase in uptake by the cancer cells as compared to the non-specific liposomes. However, the VCAM-1- targeted liposomes were localized to the tumor vasculature, as compared to the non-specific liposomes displaying a much lower degree of localization in the tumor vasculature. This was found through fluorescence microscopy of tumor sections where immunoliposomes were found to be colocalized with endothelial cells. Tumor accumulation of VCAM-1 directed immunoliposomes was evident at 30 min (91.7% colocalized) and at 24 h (73.1% colocalized), as compared to non-targeted liposomes (30.3 and 23.7% respectively). The lower fluorescence intensity of the non-targeted liposomes in the tumors could be related to the high degree of extravasation and dilution of liposomes throughout the tumor (Gosk et al, 2008).

2.3.4 Matrix metalloproteinases

Similar to the αvβ3 integrin and VCAM-1, the matrix metalloproteinases (MMPs) are targets that interact with the ECM. MMPs are a family of structurally related zinc-dependent endopeptidases capable of degrading the ECM (Vihinen et al, 2005). As an essential physiological component for tissue repair, morphogenesis, and angiogenesis, MMPs also play a role in the pathological conditions of rheumatoid arthritis, periodontitis, autoimmune blistering of the skin, tumor invasion and metastasis (Vihinen et al, 2005). The main MMP target for nanoparticulate systems has been the membrane type 1 matrix metalloproteinase (MT1-MMP), an activator of MMP-2. MMP-2, belonging to family MMP, is essential for angiogenesis as it hydrolyzes Type IV collagen, a major component of the basement membrane (Kondo et al, 2004). MT1-MMP plays a critical role in ECM degradation, endothelial cell invasion and migration, formation of capillary tubes, and recruitment of accessory cells (Genis et al, 2006).

In using MT1-MMP as a target, there are additive antitumor effects for tumor cells and endothelial cells of angiogenesis (Atobe et al, 2007). Functionally, MT1-MMP also cleaves the αvβ3 integrin, enhancing the ligand binding activity of this integrin. By targeting MT1-MMP, the ligand binding activity of the αvβ3 integrin can be limited, contributing to the value of MT1-MMP as a target (Deryugina et al, 2000). A prime example of targeting MT1-MMP involves Fab222-1D8′ fragments of anti-human MT1-MMP monoclonal antibody conjugated to doxorubicin immunoliposomes, targeted to tumor-bearing mice inoculated with HT1080 cells (Hatakeyama et al, 2007). The immunoliposomes, which had approximately 40 Fab′ fragments per liposome, were administered when the tumor was between 1000 and 3000 mm^3. After twelve days, the non-targeted liposome treatment showed a decreased tumor volume in only one out of six mice, and three of six mice died, presumably by the side effect of doxorubicin-filled non-targeted liposomes. However, in case of targeted liposomes, tumor volume decreased by a factor of two by the twelth day, and only one mouse experienced notable body weight change and no deaths occurred.

2.3.5 Human epidermal receptor

The human epidermal receptor (HER) family of receptor tyrosine kinases offers two highly upregulated targets on tumor cell surfaces. These receptor tyrosine kinases, epidermal growth factor receptor (EGFR) and human epidermal receptor-2 (HER-2), are known to mediate a cell signaling pathway for growth and proliferation in response to binding of growth factor ligand (Laskin et al, 2004). As a result, EGFR and HER-2 are most heavily researched for cancer therapeutic applications. EGFR has six known endogenous ligands: EGF, transforming growth factor-α (TGF-α), amphiregulin, betacellulin, heparin-binding EGF (HB-EGF), and epiregulin (Laskin et al, 2004). A current cancer treatment that targets EGFR is the monoclonal antibody Cetuximab® (Imclone Systems Inc., New York, NY and Bristol- Myers Squibb, Princeton, NJ), which targets the extracellular domain of EGFR and small-molecule inhibitors of tyrosine kinase activity (Grunwald and Hidalgo, 2003). One study showed that boronated immunoliposomes with conjugated Fab′ fragments of Cetuximab® mAb delivered ~8 times more boron (510 μg of 10B to 109 cells) to EGFR positive cells (F98EGFR) than non-targeted IgG immunoliposomes (61 μg of 10B to 109 cells) (Pan et al, 2007).

Tada et al. have tracked the process of tumor delivery using HER-2 (Trastuzumab) targeted quantum dots, including intravenous circulation, extravazation, binding to HER-2 on the cell membrane, endocytosis from the cell membrane to the perinuclear region and movement within the perinuclear region (Nobs et al, 2006). HER-2 is currently being used to target breast, lung, and ovarian cancer (Harries et al, 2002). Kirpotin et al. studied the mechanism by which anti-HER-2 Fab′- immunoliposomes interacted with the tumor tissue in vivo (Kirpotin et al, 2006). Specifically, the intracellular and biodistribution of 80-100 nm PEGylated liposomes conjugated with more than 20 targeting antibody fragments per liposome were investigated in mice xenograft models. Significantly higher intracellular accumulation was observed with targeted liposomes in xenografts of the HER-2 overexpressing BT-474 tumors compared to MCF-7 tumors.

2.3.6 Transferrin receptors

Due to presence of an increased number of transferring receptors on metastatic and drug resistant cells compared to normal healthy cells, transferrin is a very pertinent target for cancer therapeutics. Transferrin is a serum non-heme iron-binding glycoprotein that helps transport iron to proliferating cells (Singh, 1999).

Mark Davis from the California Institute of Technology has recently become the forerunner in transferrin targeting using self-assembled transferring-polymer-drug conjugates to treat malignant cells. Transferrin-PEG-admantane (Tf-PEG-AD) conjugates synthesized for nanoparticle modification have been used to target malignant tumors including Ewing's sarcoma (Bellocq et al, 2003; Hu-Lieskovan et al, 2005). The holo-Tf treatment resulted in the highest binding affinity, while the Tf-PEG-AD and Tf-(PEG-AD) 2 yielded lower binding affinities due to their oxidized state. As a result, a different synthesis of Tf-PEG-AD was utilized to improve the binding affinity to the transferrin receptor. The difference in receptor binding between free ligands and ligands formulated with particles could be attributed to the multiple interactions between each ligand-modified particle and cell surface receptors. Most importantly, the Tf-particle formulations were tested for gene delivery to the suspension cells K562. Formulations of 0.05% Tf-PEG-AD demonstrated a 4-fold increase in transfection compared to AD-PEG-particles (with 0.0% Tf-PEG-AD). The enhanced transfection was attributed to transferrin-mediated uptake, as the presence of excess transferrin added to 0.05% Tf-PEG-AD particles eliminated the transfection enhancement (Bellocq et al, 2003).

Davis et al. have also performed a novel study of small interfering RNA (siRNA) delivery in non-human primates using transferrin-conjugated liposomes (Heidel et al, 2007). The efficacy of these transferrin-conjugated liposomes had proven to be effective in metastatic mouse models of Ewing's sarcoma, and consequently, the safety of the administration of these particles in non-human primates was the focus of this study (Hu-Lieskovan et al, 2005). Transferrin-conjugated liposomes co-encapsulating doxorubicin and verapamil (Tf-L-DOX/VER) have shown to effectively overcome multi-drug resistance (Wu et al, 2007). Cellular uptake of Tf-L-DOX/VER had 5.2 and 2.8 times greater cytotoxicity (IC50=4.18 μM) than nontargeted liposomes with doxorubicin and

verapamil (IC50=21.7 μM) and Tf-conjugated liposomes loadedwith doxorubicin alone (IC50=11.5 μM) in a chronicmyelogenous leukemia cell line (K562 cells).

2.4 Nanoparticle

The term 'nanoparticle' may be defined as a submicron drug carrier system which is generally (but not necessarily) composed of polymer. The polymer used may or may not be biodegradable even if the polymer biodegradability appears a main characteristic for drug delivery carriers. As a function of the morphological and structural organization of the polymer, we distinguish the 'nanosphere' which is a nanoparticle system with a matrix character and constituted by a solid core with a dense polymeric network, and the 'nanocapsule' which is formed by a thin polymeric envelope surrounding an oil or waterfilled cavity. Nanocapsules may, thus, be considered as a 'reservoir' system. Practically, the nanoparticles have a size around 200 nm and the drugs or other molecules may be dissolved into the nanoparticles, entrapped, encapsulated and/or adsorbed or attached. Figure 2.2 depicts various nanocarrier based drug delivery carrier systems used for effective drug delivery research.

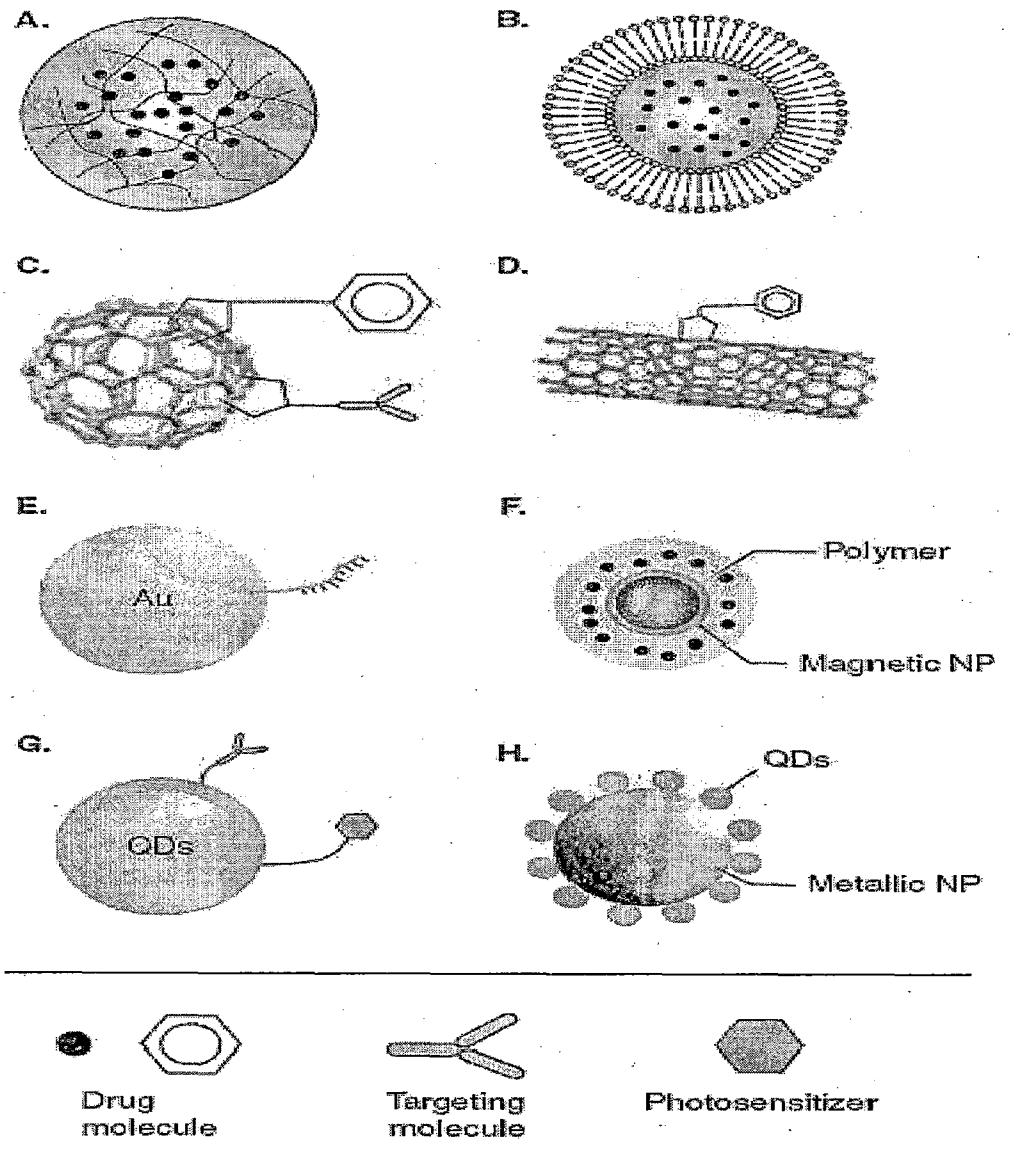
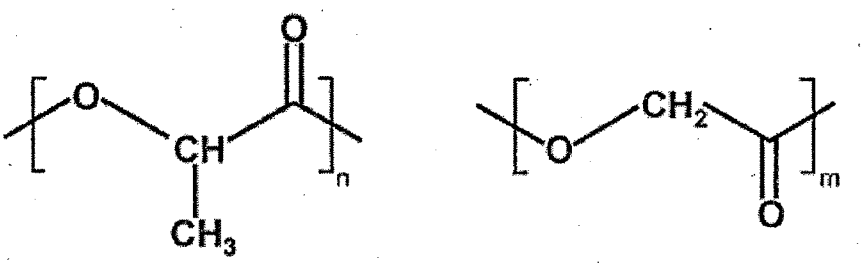


Figure 2.2: Various nanomaterial-based drug delivery platforms. A. Polymeric nanoparticles/micelles B. Liposome C. Buckyball D. Carbon nanotube E. Colloidal gold nanoparticle F. Magnetic nanoparticle G. Quantum dots H. Multifunctional nanoparticle with metallic nanoparticle core (metallic nanoparticle) and semiconductor quantum dots surrounding the shell. Drug molecules can be attached to these carrier systems through

encapsulation, mixing, covalent conjugation or electrostatic and affi nity interactions (Jiang et al, 2007).

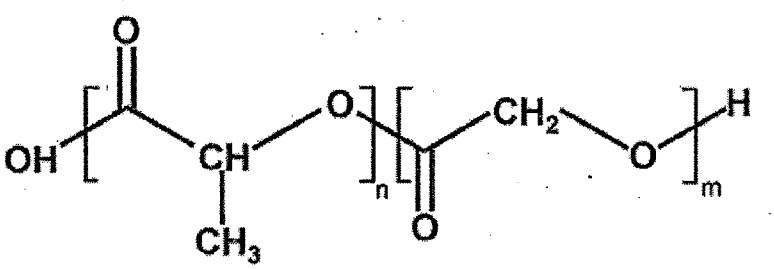
2.4.1 PLGA Nanoparticle

Recently, nano-sized drug delivery systems (DDS) especially biocompatible and biodegradable polymer nanoparticles have attracted considerable interest since they can offer a suitable means of delivering small molecular weight drugs, proteins or genes to a targeted tissue or organ (Moghimi et al, 2001; Zweers et al, 2003). Nanoparticles are colloidal systems that have size typically in the range of 10-1000 nm in diameter, and drug can be entrapped in, adsorbed or chemically coupled onto the polymer nanoparticle matrix (Labhasetwar et al, 1997). On the other hand, a number of polymers have been investigated for formulating biodegradable nanoparticles, such as polylactide (PLA), poly(3-caprolactone) (PCL) and poly(lactide-co-glycolide) (PLGA) (Fig 2.3). They are biocompatible and biodegradable polymers approved by FDA and have been studied extensively (Rouzes et al, 2000; Berton et al, 1999).



Poly(lactic acid)

Poly(glycolic acid)



Poly(lactic-co-glycolic acid)

Figure 2.3: Molecular structure of lactide and glycolide based biodegradable polymer

PLGA is one of the most successfully used biodegradable nanosystem for the development of nanomedicines because it undergoes hydrolysis in the body to produce the biodegradable metabolite monomers, lactic acid and glycolic acid. Since the body effectively deals with these two monomers, there is very minimal systemic toxicity associated by using PLGA for drug delivery or biomaterial applications. PLGA nanoparticles have been mostly prepared by emulsification–diffusion (Sahana et al, 2008), solvent emulsion–evaporation (Zambaux et al, 1999), interfacial deposition (Pinto Reis et al, 2006) and nanoprecipitation method (Barichello et al, 1999) (Fig. 2.4). Generally in emulsification–diffusion method, the PLGA polymers are dissolved in organic solvent, poured and separated in aqueous phase having stabilizer and subsequently emulsified by homogenizer. In solvent evaporation method, the polymers are dissolved in volatile organic solvent and poured into continuously stirring aqueous phase with or without emulsifier/stabilizer and sonicated. Interfacial deposition methods have been used for the formation of both nanocapsule and nanospheres. The nanoparticles are synthesized in the interfacial layer of water and organic solvent (water miscible) and finally the nanoparticles are separated by centrifugations (Pinto Reis et al, 2006). Most commonly used method for the preparation of PLGA nanoparticles is nanoprecipitation. Polymer dissolved in acetone is added drop-wise into continuously stirring aqueous phase with or without emulsifier/stabilizer and consequently organic phase is evaporated under reduced pressure (Fig. 2.4).

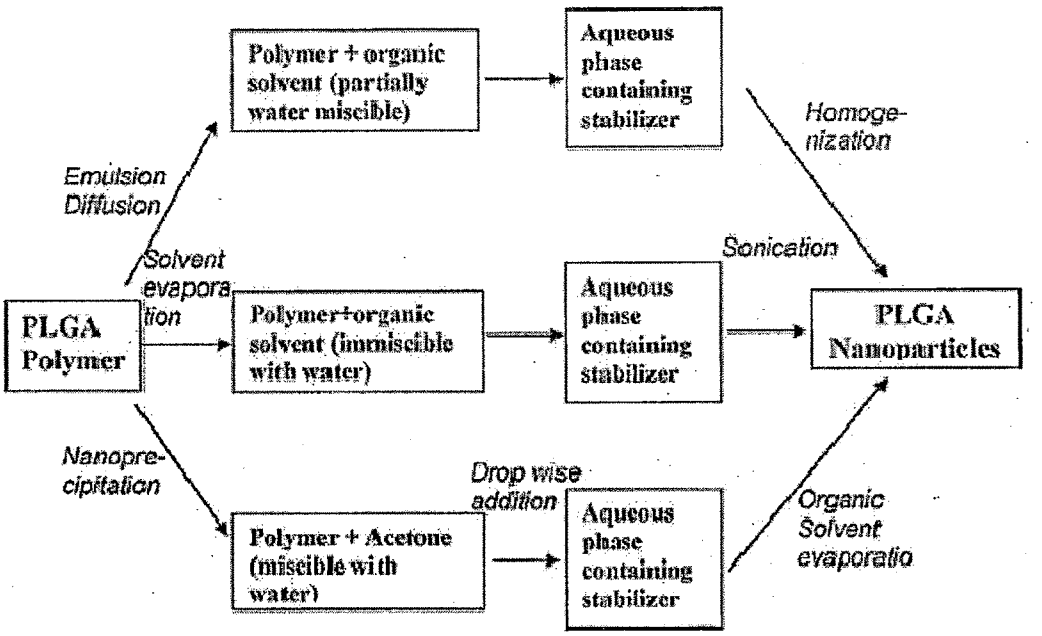


Figure 2.4: Different method for preparation of PLGA nanoparticles: PLGA nanoparticles were synthesized by emulsion diffusion, solvent evaporation and nanoprecipitation methods. (Kumari et al, 2010)

PLGA nanoparticles have been used to develop the proteins and peptides nanomedicine, nano-vaccines, nanoparticles based gene delivery system, nano-antigen and growth factor, etc. (Soppimath et al, 2001, Carrasquillo et al, 2001). Surface modification of PLGA, drug encapsulation methods and particle size, additives added during formulation, molecular weight of drug, ratio of lactide to glycolide moieties has strong influence on the release and effective response of formulated nanomedicines (Mittal et al, 2007). The acidic nature of PLGA monomers is not suitable for some sensitive drugs or bioactive molecules (Kumar et al, 2004). However, the approaches to overcome these problems have been developed. PLGA nanomedicine formulations are blended with alginate, chitosan, pectin (Liu et al, 2004), poly(propylenefumarate) (Hedberg et al, 2004) polyvinylacohol (Patil et al, 2004), poly(orthoester), etc. (Wang et al, 2004). The approval of PLGA has been granted by US Food and Drug Administration (USFDA) for

human use and nanomedicines (Di Toro et al, 2004). Many drugs of various diseases are formulated and commercialized in the market (Mundargi et al, 2008). (Give some examples) Many encapsulants have been successfully entrapped into/or adsorbed to PLGA nanoparticles.

PLGA is approved by FDA for therapeutic use in humans. Various preparation methods have been optimized for PLGA nanoparticles synthesis and numerous cancer related drugs have been incorporated in PLGA (Hans, 2002). These loaded nanoparticles protect poorly soluble and unstable payloads from the biological milieu and are small enough for capillary penetrations, cellular internalization and endosomal escape (Soppimath et al, 2001). Furthermore, their surface is modified for targeted delivery of molecules to tumor or other tissues (Nobs et al, 2004). The larger size of PLGA nanoparticles is advantageous as multifunctional imaging and probes which incorporate encapsulated cancer drug, release, imaging, and targeting in a single nanoparticles platform (Torchilin, 2006).

The performance of these nanoparticles is not completely satisfactory and great effort is needed to improve its physiochemical properties and synthesis process. The properties of nanoparticles as precursor of good nanomedicine are particle size, size distribution, surface morphology, surface chemistry, surface charge, surface adhesion, surface erosion, interior porosity, drug diffusivity, drug encapsulation efficiency, drug stability, drug release kinetics and hemodynamic. The surface charge of the nanoparticles is important for the cellular internalization of the NPs, clustering in blood flow, adherence, and interaction with oppositely charged cells membrane (Feng et al, 2004). PLGA nanoparticles are frequently used for the encapsulation of various cancer related drugs and their successful delivery in vivo. The cancer related drug paclitaxel, doxorubicin, 5-fluorouracil, 9-nitrocamptothecin, cisplatin, triptorelin, dexamethasone, xanthone, etc., have been successfully encapsulated on PLGA nanoparticles (Kumari et al, 2010). The mechanism of action of these drugs, encapsulation mechanism, encapsulation efficiency, peculiar characteristic for encapsulation and drug release mechanisms of this cancer therapeutics are briefly described.

2.4.2 PBCA Nanoparticle

Poly(alkylcyanoacrylates) (PACA) NP systems are attractive delivery system among all NP because the methods of preparation are generally simple and easy to scale-up. The fate of colloidal particles after intravenous (IV) administration is determined by a combination of biological and physicochemical events that need to be considered in the design of efficient drug carrier systems. After IV administration, all colloidal systems, indeed, dramatically interact with plasma proteins, especially with immunoglobulins, albumin, elements of the complement, fibronectin, etc. This process, known as 'opsonization' is crucial in dictating the subsequent fate of the administered colloidal particles. Thus, colloidal particles that present hydrophobic surface properties are efficiently coated with plasma components and rapidly removed from the circulation, since the macrophages of the liver and the spleen own their specific receptors for these opsonins. However, colloidal particles that are small and hydrophilic enough can escape, at least partially, from the opsonization process and consequently remain in the circulation for a relatively prolonged period of time. Moreover, the concept of 'steric hindrance' has been applied to avoid the deposition of plasma proteins either by adsorbing at the surface of the so-called 'stealth' colloids (Peracchia et al, 1998; Peracchia et al, 1999a).

In general, the PEGylated PBCA nanoparticles showed a reduced total amount of proteins adsorbed as compared to their conventional counterparts. The adsorption of opsonic proteins such as fibrinogen and IgG was also decreased because of the presence of PEG chains on the particle surface (Peracchia et al, 1999a, Peracchia et al, 1999b). This prolonged half-life in the blood compartment allows these nanoparticles to selectively extravasate in pathological sites, like tumors or inflamed regions with a leaky vasculature. This leads to the enhanced permeability and retention (EPR) effect which results in a higher drug accumulation in tumor tissues than in plasma and in other tissues (Noguchi et al, 1998) (Fig 2.5). So, these 'stealth' nanoparticles are very promising in their capabilities of targeted drug delivery. In addition, active targeting can be achieved by attaching a specific ligand (such as a monoclonal antibody) onto the surface of the

colloidal particle, preferentially at the end of the PEG molecules, since the targeted colloidal particles will be much more efficient if they are also sterically stabilized. An *in vivo* study has evidenced the ability of nanoparticles composed of chitosan-PEG and attached to an antibody directed against transferring receptor, to reach the target of healthy animals after IV injection (Maeda et al, 2000).

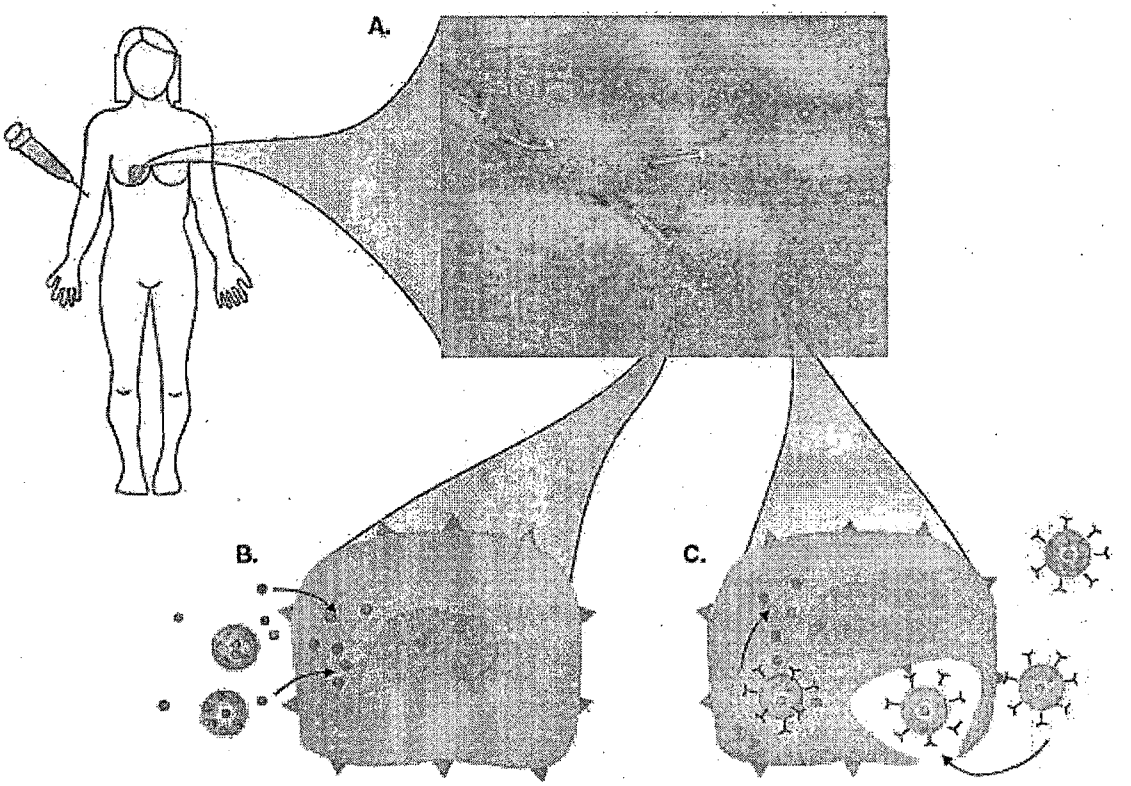


Figure 2.5: Nano drug delivery systems *in vivo*. A. Intravenously administered nano-drug delivery systems flow within the bloodstream. B. Nano-drug delivery system passive targeting is achieved due to heterogeneous vasculature formation found in pathological processes such as tumors, resulting in enhanced permeation retention effects. The accumulation of nano-drug delivery systems at the targeted site results in enhanced drug payload. C. In contrast, active targeting is achieved using recognition molecules attached

to nano-drug delivery system surfaces for specific ligand-receptor mediated interactions (Jiang et al, 2007).

The PBCA polymers are a family of biodegradable polymers which have been extensively used to prepare nanoparticles for drug delivery. The degradation speed by esterases depends on the length of the alkyl chain (Vauthier et al, 2003). Another advantage of PBCA nanoparticles is that only these polymers are able to overcome the multidrug resistance (MDR) (Brigger et al, 2002; Vauthier et al, 2003). MDR allows tumors to evade chemotherapy due to the overexpression of P glycoprotein (Pgp). MDR is one of the greatest problems in chemotherapy. A complex mechanism has been proposed to explain the efficacy of doxorubicine (DOX)-loaded PBCA nanoparticles on tumor treatment by the formation of an ion pair between the DOX and the degradation product of PBCA which can diffuse across tumor cell membrane. In addition, PBCA polymers allow the modification of the surface of nanoparticles by coating of surfactants (Kreuter et al, 1995) or by covalent linkage of PEG chains (Peracchia et al, 1999b) in order to obtain 'stealth' nanoparticles.

Poly(butylcyanoacrylate) (PBCA) nanoparticles are prepared from butylcyanoacrylate (BCA) monomer by anionic polymerization in water at low pH in the presence of small amounts of surfactant (e.g., poloxamer 188, polysorbate 80, dextran 70,000). The polymerization seems to proceed by initial addition of an OH- anion to the strongly activated C=C double bond followed by linear chain elongation with consecutive addition of cyanoacrylate monomers and eventual termination of the growing chains by uptake of a proton. The aggregation of the resulting cyanoacrylate oligomers which are of varying chain lengths leads to the formation of nanoparticles.19 The better condition to prepare uniform nanoparticles in size and mass distribution is to control pH condition.

In earlier studies done by Kreuter et al., molecules such as dalargin (Schroeder et al, 1998), loperamide (Alyautdin et al, 1997) and tubocurarine (Alyautdin et al, 1998) have been loaded onto nanoparticles with the aim of its delivery to the brain. After peripheral administration, these molecules themselves do not exhibit any therapeutic effect because

they do not diffuse through the BBB. But, when dalargin, loperamide or tubocurarine were adsorbed onto the surface of PBCA nanoparticles further coated with a surfactant, brain delivery was observed. Moreover, poloxamer 188 (Pluronic F68) failed to enhance the delivery of dalargin (Petri et al, 2007). Ambruosi et al. (2006) compared the antitumoral effect of DOX-loaded PBCA nanoparticles coated with different surfactants like PS80, poloxamer 188, and poloxamine 908 in a rat glioma model evidencing the better increase of survival times with PS80 coating. The biodistribution of DOX-loaded PBCA nanoparticles coated with PS80 has been performed after IV injection into healthy rats and glioblastoma-bearing rat (Ambruosi et al, 2005). These studies showed a decrease of reticuloendothelial system (RES) uptake with PS80 coating.

The size of the nanoparticles is also an important factor to achive long circulation and site specific localization. Methotrexate-loaded PBCA nanoparticles and coated by PS80 of different sizes have been IV injected into rats. The results have shown that the nanoparticles of 70 nm are able to deliver into tumor more methotrexate than nanoparticles with higher sizes (170, 220 and 345 nm) which exhibit no significant difference (Gao et al, 2006) Recently, a double coating by PS80 and PEG 20,000 on dalargin loaded PBCA nanoparticles allowed the observation of an antinociceptive effect by the tail-flick test following the oral administration to mice (Das and Lin, 2005).

2.4.2.1 Preparation and Characterization of PEGylated PBCA NPs

To obtain 'stealth' nanoparticles, block PEG-PACA copolymers have been synthesized by initiating the polymerization of alkylcyanoacrylates either by monomethoxy (MeO)-PEG-triphenylphosphine or by triphenylphosphine-PEG-triphenylphosphine, leading to diblock MeO-PEG-PBCA or triblock MeO-PBCA-PEG-PBCA-OMe linear copolymers, respectively (Choi et al, 1995). Branched PEG-PBCA copolymer is obtained by the Knoevenagel reaction of block copolymers, involving the condensation of a cyanoacetate derivative with formaldehyde. This MeO or monoamino poly(PEG- cyanoacrylate-cohexadecylcyanoacrylate) (PEG-PHDCA) copolymer allows the production of very stable nanospheres and avoids the use of the triphenylphosphine group, which may be toxic (Peracchia et al, 2003). With these copolymers, nanoparticles are usually prepared

by nanoprecipitation or emulsification solvent evaporation. Nanoprecipitation is based on the formation of colloidal polymer particles during phase separation induced by the addition of a nonsolvent of the polymer to a dilute polymer solution. This method usually consists of dissolving the polymer in an organic solvent miscible with water (e.g., acetone) and mixing it with an aqueous solution, leading to the precipitation of the polymer and the formation of nanospheres spontaneously. The organic solvent is removed from the suspension by rota-evaporation. In emulsification-solvent evaporation method, first of all, the polymer is dissolved in a water-immiscible organic solvent such as methylene chloride or ethyl acetate, and then the polymer solution is emulsified in an aqueous phase by help of a high-pressure homogenizer or a microfluidizer to produce emulsion droplets of very small size. Finally, the polymer solvent is evaporated, inducing polymer precipitation as nanospheres (Brigger et al, 2002).

The particle size may also be reduced by increasing the concentration of surfactant or stabilizing agent used for their preparation, such as cholic acid, sodium salt, poly(vinyl alcohol) or Pluronic F68 (polyoxyethylene-polyoxypropylene block). For example, the size of PEGylated PBCA nanospheres varied from 50 to 300 nm as a function of the concentration of Pluronic F68 (1–0.5%) used as stabilizing agent (Peracchia et al, 1999a). In spite of washing the nanoparticles, traces of surfactants can remain associated with the nanosphere surface, hence influencing their physicochemical characteristics as the zeta potential. The presence of PEG chains on the nanoparticle surface was found to dramatically change their zeta potential. PEG-PHDCA nanoparticles prepared from mPEG-co-HDCA copolymer showed zeta potential value close to neutrality (–5/–10 mV), compared with non-PEGylated nanoparticles (-25/ -30 mV) (Peracchia et al, 1998). In general, the zeta potential increases (from negative charge to neutral charge) when the PEG density and length increased. On the contrary, phagocytosis by polymorphonuclear cells (PMN) uptake decreases with the increase in the PEG content of the nanoparticles (Gref et al, 2000). Thus, the length and density of PEG chains is shown to play an important role in the surface charge and the PMN uptake of nanoparticles. The hydrophilicity of nanoparticles, due to the PEGylation could be estimated using hydrophobic interaction chromatography. In a column filled with propylamine-agarose

gel, PEG-PHDCA nanoparticles can be eluted easily through the gel phase, whereas hydrophobic PHDCA nanoparticles are removed only by washing with TritonX-100 (Peracchia et al, 1998). However, this method did not allow the separation of nanoparticles with different hydrophilic material coatings.

2.5 Bisphosphonates

After more than three decades of research and development, bisphosphonates have now become indispensable for the treatment of both benign and malignant bone disease (Fleisch, 1997). Bisphosphonates are metabolically stable analogues of pyrophosphate (P-O-P), an endogenous regulator of bone mineralization, in which the central oxygen atom is replaced with a carbon atom (P-C-P). Extensive chemical research programmes have produced a wide range of molecules with various substituents attached to the carbon atom (Shinoda et al, 1983). The early compounds contained simple substituents (H, OH, Cl, and CH_3) and lacked a nitrogen atom. Subsequently, more complex compounds were produced; common substituents being a hydroxyl group together with nitrogen-containing aliphatic side chains or heterocyclic rings (Fig. 2.6). Despite this structural diversity, the common, highly charged bisphosphonate group dominates the physicochemical properties of the entire class of compounds and is responsible for their high binding affinity to the hydroxyapatite in mineralized bone (Jung et al, 1973).

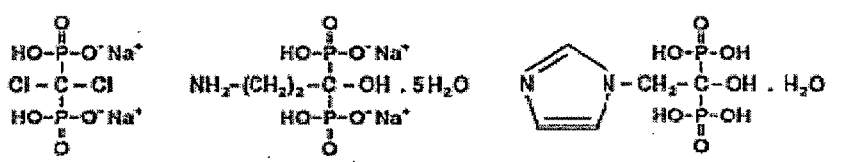


Figure 2.6: Chemical structure of three representative bisphosphonate compounds.

2.5.1 Bisphosphonates as anticancer drug

Recently, considerable progress have been made in elucidating the molecular mechanism of action of bisphosphonates and, not surprisingly, it has been found to differ between the 2 structural subgroups (Rogers et al, 2000). Etidronate and clodronate appear to form

cytotoxic ATP-analogues after internalization by mammalian cells. By contrast, the nitrogen-containing bisphosphonates inhibit farnesyl diphosphate synthase, a key enzyme in the mevalonate pathway, thereby reducing the prenylation of small GTP-binding proteins that are essential for normal cell function and survival. Irrespective of the molecular mechanism of action, bisphosphonate treatment results in the inhibition of osteoclastic activity followed by the induction of apoptosis.

Despite this potent cytotoxic effect on osteoclasts in bone, bisphosphonates are remarkably nontoxic to other organs and are very well tolerated clinically (Fleisch et al, 1998). This may be explained by the rapid accumulation of bisphosphonate in bone, resulting in a very short plasma half-life and low exposure of visceral tissues. Data are now beginning to accumulate showing that bisphosphonates may also affect cell types other than the osteoclast. Several studies have clearly demonstrated that bisphosphonates are cytostatic to tumor cells in vitro, induce apoptosis, inhibit cell adhesion and interfere with the metastatic process (Goltzman et al, 2001). Bisphosphonate bound to bone could also be released during this process and thus exert cytostatic and proapoptotic effects not only on osteoclasts but also on tumor cells and stromal cells in bone metastases.

Although still equivocal at present, some of the in vivo data indicate that the more potent nitrogen-containing bisphosphonates can reduce tumor load and induce tumor cell apoptosis in animal models of bone metastases. Moreover, newly emerging data show that some bisphosphonates are also angiostatic and can modulate the immune response properties that could contribute to the overall antitumor efficacy.

2.5.2 Inhibition of tumor cell proliferation and induction of apoptosis in vitro

Several in vitro studies have shown that bisphosphonates exert direct cytostatic and proapoptotic effects on a variety of human tumor cell lines (myeloma, breast, prostate, and pancreas) in a concentration and time dependent manner (Shipman et al, 1997). The pioneering studies by Shipman et al. were the first to demonstrate antitumor activity of bisphosphonate on myeloma cell lines in vitro (Shipman et al, 1998). The results have subsequently been confirmed and extended by several groups using a variety of myeloma

cell lines and bisphosphonates (Aparicio et al, 1998). Overall the results clearly demonstrated that various N-BPs (zoledronic acid, pamidronate and incadronate) decrease myeloma cell proliferation and induce apoptosis, whereas the nonnitrogen containing compound clodronate has little or no effect. Addition of geranylgeraniol and farnesol, two intermediates of the mevalonate pathway, prevents the bisphosphonate-induced apoptosis and partially reverses the cell cycle arrest, consistent with the proposed mechanism of action of the N-BPs. Bisphosphonate induced cytostasis was maintained even in myeloma cells subjected to the strong proliferative stimulus of IL-6 (Aparicio et al, 1998). Interestingly, the apoptotic effect (but not the cytostatic one) of zoledronic acid could be prevented by transfection of IM-9 myeloma cells with a vector expressing the antiapoptotic protein bcl-2. The cytostatic and apoptotic effects of bisphosphonates observed on myeloma cell lines have been confirmed with primary bone marrow stromal cells isolated from patients with multiple myeloma (Derenne et al, 1999).

In addition to their direct antiproliferative and proapoptotic effects on myeloma cells, bisphosphonates may also affect the proliferation and survival of myeloma cells in vivo by inhibiting the release of growth factors from osteoblasts and bone marrow stromal cells. Secretion of IL-6 by human bone marrow stromal cells in culture is decreased by bisphosphonate treatment and this effect is not reversed by addition of exogenous IL-6 (Savage et al, 1996). Both zoledronic acid and pamidronate inhibit the IL-1-stimulated production of matrix metalloproteinase-1 by human bone marrow stromal cells in vitro, but paradoxically these 2 compounds appear to upregulate matrix metalloproteinase- 2 (Derenne et al, 1999).

2.5.3 Inhibition of tumor cell adhesion and invasion

Tumor cell adhesion and invasion are essential steps in the metastatic process. Bisphosphonate treatment inhibits the binding of human breast and prostate cancer cells to mineralized and unmineralized matrices regardless of whether the tumor cells or the binding matrices are treated with bisphosphonate (Magnetto et al, 1999). The process of tumor cell invasion through extracellular matrix is also potently inhibited in vitro by bisphosphonates, although cell motility per se is not as markedly affected (Boissier et al,

2000). It is interesting to note that some of these effects were observed with very low bisphosphonate concentrations in the range 10^{-12}-10^{-6} M, the lowest concentrations reported for a biologic response to a bisphosphonate, and again they could be enhanced by the addition of taxoids (Virtanen et al, 2002). The mechanism by which nitrogen-containing bisphosphonates inhibit tumor cell adhesion to bone matrix remains unknown; modulation of cell adhesion molecules such as cadherin, laminin, and the integrins is probably involved.

Because tumor cell invasion also requires digestion of the basement membrane by proteases, this step in the metastatic cascade is another potential target for attenuation. Bisphosphonates inhibit the activity of several matrix metalloproteinase enzymes (MMP-1, -2, -3, -7, -8, -9, -12, -13, -14) in vitro with IC50 values in the range of 50–150 μM (Teronen et al, 1997). The mechanism appears to be chelation of divalent cations from the enzyme active site since addition of excess zinc reverses the inhibition (Boissier et al, 2000).

2.5.4 Effects on the secretion of growth factors and cytokines

In the complex microenvironment of a bone metastasis, many cell types and growth factors are present that could influence tumor cell proliferation. Thus, the high local bisphosphonate concentration in bone may exert effects not only on osteoclasts and tumor cells, but also on other cells such as osteoblasts, bone marrow stromal cells, and monocytes. Bone is a rich source of growth regulatory substances (TGF-β, BMPs, FGFs, PDGFs, IGFs, etc.) that are released during bone resorption and can stimulate tumor cell proliferation (Guise TA, Mundy, 1998). Moreover, tumor cells secrete PTHrP, which potently stimulates osteoclasts to resorb bone and release yet more bone-bound growth factors. In animal models of bone metastases, the pivotal role of PTHrP and TGF-β in disease progression has been elegantly demonstrated by the use of neutralizing antibodies to PTHrP and tumor cells with an inactive TGF-β receptor (Guise et al, 1998). By inhibiting osteoclastic activity, bisphosphonates can reduce the local release of growth factors from bone and break this self-perpetuating stimulatory mechanism, resulting in the retardation of tumor growth (Sahni et al, 1993).

2.5.5 Inhibition of angiogenesis

Angiogenesis and neovascularization are essential for the growth and survival of metastases. The potent, imidazole-containing bisphosphonate zoledronic acid has been shown to markedly inhibit the in vitro proliferation of human umbilical vein endothelial cells induced by bFGF, VEGF, or serum with IC50 values of 4-5 μM, whereas pamidronate had no effect at similar concentrations (Wood et al, 2002). Preliminary findings indicate that low micromolar concentrations of zoledronic acid inhibit endothelial cell adhesion to vitronectin and significantly decrease αVβ3 and αVβ5 integrin expression (Bonjean et al, 2002). When administered to mice for 5 days, zoledronic acid at a dose of 1-100 μg/kg/day s.c. potently inhibited the angiogenic response induced by subcutaneous implants loaded with bFGF and had a weaker effect against VEGF stimulation (Wood et al, 2002). This antiangiogenic effect of zoledronic acid has been independently confirmed in two other in vivo models: benign prostate hypotrophy in castrated rats and the murine 5T2 model of multiple myeloma (Boissier et al, 2002; Croucher et al, 2002). Castration of rats induces vascular regression and hypotrophy of the ventral prostate, an effect that can be reversed by subsequent administration of testosterone. Treatment of these rats with testosterone combined with zoledronic acid (1-100 μg/kg/day) inhibited the testosterone induced vascular regrowth in the rat ventral prostate. In the 5T2 myeloma model, treatment of tumor-bearing mice with zoledronic acid (120 μg/kg twice a week) reduced microvessel density in the myelomatous bone lesions.

2.5.6 Inhibition of bone metastases in vivo

Several studies during the past two decades have clearly demonstrated that a variety of bisphosphonates can reduce tumor-induced osteolysis in animal models of breast (Hall DG, Stoica, 1994), prostate (Pollard and Luckert, 1985), and bladder cancer (Nemoto et al, 1987), as well as multiple myeloma (Radl et al, 1985). By inhibiting osteoclast-mediated bone resorption, bisphosphonates decrease the release of tumor-promoting growth factors from bone and delay the further progression of bone metastases. As a result, not only is osteolysis reduced, but there is also a reduction in both the number and

size of tumors in bone. Zoledronic acid (Croucher et al, 2002) has been shown to inhibit tumor-induced osteolysis, as measured by a variety of radiographic, histologic, and histomorphometric techniques.

2.5.7 Evidence of antitumor efficacy in vivo

In an early study with the murine 5T2 myeloma model, it was noted that pamidronate appeared to be cytotoxic to the myeloma cells (Radl et al, 1985). Later studies demonstrated increased apoptosis of tumor cells in osteolytic lesions in mice treated with pamidronate, ibandronate and zoledronic acid (Yaccoby et al, 2002). The lowering of serum levels of tumor markers such as prostate-specific antigen and myeloma paraprotein in tumor-bearing animals treated with zoledronic acid is further indirect evidence of an antitumor effect in vivo (Croucher et al, 2002). Bisphosphonate treatment has occasionally prolonged overall survival or disease-free survival, but the effects were modest (Croucher et al, 2002). Changes in visceral metastases and soft tissue xenografts have been inconsistent and are difficult to interpret.

2.5.8 Immunomodulatory effects of bisphosphonates

Emerging data indicate that nitrogen-containing bisphosphonates can exert a broad spectrum of immunomodulatory effects that could be beneficial in a variety of disease states. In the clinical literature, there are already intriguing case reports suggesting that N-BPs can modulate the immune response. The administration of pamidronate to several AIDS patients was associated with a rapid reversal of cachexia and a marked increase in TGF-β1 secretion by cultured peripheral blood mononuclear cells (Postlethwaite et al, 1999). A recent clinical study indicated that bisphosphonate treatment can also suppress the immune response. In a controlled, randomized trial with ibandronate in kidney transplant patients, the rejection rate in the ibandronate-treated group was reduced to half that of the controls (11 vs. 22, $P = 0.009$) (Grotz et al, 2001). Clearly, bisphosphonates can exert a variety of complex effects on the immune system and the entire area warrants further attention.

2.6 Docetaxel as anticancer drug

It is becoming clear that certain chemotherapeutic agents such as docetaxel have multiple effects within a tumor and interact with a variety of processes involved in cancer cell growth and metastasis. Docetaxel is a second-generation taxane derived from the needles of the European yew tree. Early in vitro studies revealed that docetaxel has a wide spectrum of antitumor activity and a number of unique preclinical characteristics compared to other chemotherapeutic agents, including the taxane paclitaxel (Bissery et al, 1995). For instance, in several murine and human tumor cell lines, docetaxel exhibited 1.3 to 12 fold greater cytotoxicity relative to paclitaxel (Ringel et al, 1991). A similar pattern emerges from its broad spectrum of activity in vivo with murine tumor models and human tumor xenografts (Bissery et al, 1995). Furthermore, unlike paclitaxel, docetaxel exhibits linear pharmacokinetics and, due to differences in drug efflux, is retained intracellularly for a longer period (Bissery et al, 1995). This preclinical promise has translated into clinical practice. Docetaxel is highly effective as monotherapy and combination therapy across a variety of tumor types, including breast, lung, ovarian, as well as head neck, gastric, and prostate carcinomas (Crown and O'Leary, 2000).

2.6.1 Molecular targets of Docetaxel

2.6.1.1 An antimicrotubule agent

The central role of microtubules in cell division and other relevant cellular functions makes them a focus for anticancer drug development (Rowinsky and Donehower, 1996). The primary mechanism of action for the taxanes (docetaxel and paclitaxel) is to promote microtubulin assembly and stabilize the polymers against depolymerization, thereby inhibiting microtubule dynamics. This mechanism of action employed by taxanes is distinct from that of other antimicrotubule agents, which prevent microtubule assembly (Rowinsky and Donehower, 1996).

Although docetaxel and paclitaxel share a mutual microtubule binding site (for which docetaxel has a higher affinity), there is evidence that they have distinct effects on microtubule dynamics (Andreu et al, 1994). This may underlie the greater potency of docetaxel as a tubulin assembly promoter and microtubule stabilizer compared to that of paclitaxel (Gueritte et al, 1991). Furthermore, preliminary data suggest that low levels of

expression of specific microtubule-associated proteins (e.g., the class II b-tubulin isotype) may correlate with higher docetaxel response rates - a potential predictive marker for docetaxel activity (Bernard et al, 2001). The consequences of blocking microtubule dynamics are complex: a number of vital cellular functions in which microtubules play a critical role are compromised. Impairment of mitotic progression leading to cell cycle arrest is considered to be a principal component of docetaxel's mechanism of action. This blocks progression of a cell through its natural division cycle and, consequently, inhibits cell proliferation.

2.6.1.2 Docetaxel influences apoptosis pathways

Disruption of microtubules not only affects progression through the cell cycle, but may also alter signaling pathways involved in processes such as apoptosis. Several signal transduction pathways may be involved in docetaxel's effects on apoptosis (Wang et al, 1999). The Bcl-2 gene family in particular appears to play a critical role in the regulation of apoptosis. Antimicrotubule agents are believed to cause inactivation of Bcl-2 function through phosphorylation (Haldar et al, 1997). Docetaxel is 10 to 100 fold more potent than paclitaxel in phosphorylating Bcl-2 and this may account for the differential pro-apoptotic activity of docetaxel compared with paclitaxel. An association of docetaxel-induced apoptosis with increase in tumor blood vessel diameter may have the beneficial secondary effect of improving delivery of other therapeutic agents (Griffon et al, 1999).

2.6.1.3 Docetaxel inhibits angiogenesis

Angiogenesis is the process by which tumors develop new capillary blood vessels (Liekens et al, 2001). The process is vital for tumor progression and is intrinsically connected with metastases (Kirsch et al, 2001). Inhibition of angiogenesis is a potential strategy in antitumor drug development, with a number of agents currently undergoing clinical investigation (Rosen, 2000). Such a strategy may have advantage with respect to toxicity and drug resistance. Docetaxel has shown to inhibit angiogenesis both in vitro and in vivo (Sweeney et al, 2001). VEGF has been shown to shield tumor cells from the antiangiogenic effects of docetaxel and VEGF antibodies can overcome the protective effect both in vitro and in vivo. In the clinic, VEGF overexpression is associated with

larger tumor size, increased metastasis, and poor prognosis in metastatic breast cancer (MBC) patients (Saaristo et al, 2000). Enhancement of the antiangiogenic properties of docetaxel through inhibition of endogenous angiogenic growth factors such as VEGF is a strategy that merits further investigation.

2.7 Combination effect of Zoledronic acid and Docetaxel

Docetaxel has been approved for the treatment of hormone and drug resistant prostate cancer patients and exhibits its cytotoxic effect due to decreased proliferation and induction of apoptosis (Berthold et al., 2008). Zoledronic acid is the most potent member of bisphosphonates that was shown to be standard therapy for patients with bone metastasized hormone refractory prostate cancer and these agents decrease skeletal related events (Saad, 2002; Saad et al., 2004). Zoledronic acid was also shown to have anti-tumoral effects on prostate cancer cells through induction of apoptosis, inhibition of cell proliferation and angiogenesis (Oades et al., 2003).

It was clear that docetaxel and zoledronic acid combination exerts a significant degree of cytotoxicity on PC-3 and DU-145 prostate carcinoma cells as compared to any agent alone. These cells are ideal models to study the effects and mechanisms of various anticancer agents since they represent very high aggressive nature of metastatic human prostate cancers. Results revealed that docetaxel and zoledronic acid are significantly cytotoxic in both PC-3 and DU-145 cells, in a dose and time-dependent manner. It was demonstrated that there was a concentration dependent increase in caspase 3/7 enzyme activity in prostate carcinoma cells exposed to zoledronic acid or docetaxel and combinations of both (Karabulut et al, 2009).

Combination therapy of ZOL with paclitaxel has already showed synergistic effects (Jagdev et al. 2001). Some researchers have also studied combination therapy of ZOL with DTX. Morgan et al. found that tumor volume was reduced by 64% with significant improvement from skeletal complication and reduced serum PSA (prostate specific antigen) level when ZOL-DTX was used in combination with mTOR inhibitor (Everolimus) in intra-tibial tumor of C4-2 CaP cancer in mice model. Results of a phase

II trial conducted on 27 patients with the combination of ZOL-DTX along with estramustine showed that 52% had a biological response with more than 50% reduction in serum PSA (Kattan et al. 2008). Eligibility criteria for the study consisted of metastatic prostate adenocarcinoma with objective progression or rising prostate specific antigen levels (PSA) despite androgen deprivation therapy.

So, it is hypothesized that the proposed ZOL functionalized docetaxel loaded nanoparticulate system may target bone metastasis with synergistic effect on metastatic tumor, thereby reducing bone complication by controlling ostreoclast activity by bisphosphonate along with reduction in further metastasis by reducing activation of growth factors by controlling ostreoclast activity and reducing occult tumor cell count in blood.

2.8 Reference:

1. Rubens RD. Bone metastases: the clinical problem. Eur J Cancer 1998;34(2):210-3.
2. Orr FW, Lee J, Duivenvoorden WC, Singh G. Pathophysiologic interactions in skeletal metastasis. Cancer 2000;88(12):2912-8 (suppl).
3. Roodman GD. Mechanisms of bone metastasis. N Engl J Med 2004;350(16): 1655-64.
4. Yi B, Williams PJ, Niewolna M, Wang Y, Yoneda T. Tumor-derived plateletderived growth factor-BB plays a critical role in osteosclerotic bone metastasis in an animal model of human breast cancer. Cancer Res 2002;62(3):917-23.
5. Prasad A, Fernandis AZ, Rao Y, Ganju RK. Slit protein-mediated inhibition of CXCR4-induced chemotactic and chemoinvasive signaling pathways in breast cancer cells. J Biol Chem 2004;279(10):9115-24.
6. Norton L, Massague J. Is cancer a disease of self-seeding? Nat Med 2006;12(8):875-8.
7. Cooper CR, Chay CH, Gendernalik JD, et al. Stromal factors involved in prostate carcinoma metastasis to bone. Cancer 2003;97(3):739-47 (suppl).
8. Romanenko A, Morell-Quadreny L, Ramos D, Nepomnyaschiy V, Vozianov A, Curr Probl Cancer, March/April 2007 95
9. Llombart-Bosch A. Extracellular matrix alterations in conventional renal cell carcinomas by tissue microarray profiling influenced by the persistent, long-term, low-dose ionizing radiation exposure in humans. Virchows Arch 2006;448(5): 584-90.
10. Keller ET, Brown J. Prostate cancer bone metastases promote both osteolytic and osteoblastic activity. J Cell Biochem 2004;91(4):718-29.
11. Schneider A, Kalikin LM, Mattos AC, et al. Bone turnover mediates preferential localization of prostate cancer in the skeleton. Endocrinology 2005;146(4): 1727-36.
12. Berruti A, Dogliotti L, Tucci M, Tarabuzzi R, Fontana D, Angeli A. Metabolic bone disease induced by prostate cancer: rationale for the use of bisphosphonates. J Urol 2001;166(6):2023-31.

13. Chirgwin JM, Guise TA. Molecular mechanisms of tumor-bone interactions in osteolytic metastases. Crit Rev Eukaryot Gene Expr 2000;10(2):159-78.
14. Mundy GR. Metastasis: Metastasis to bone: causes, consequences and therapeutic opportunities. Nat Rev Cancer 2002;2(8):584-93.
15. Saito H, Tsunenari T, Onuma E, Sato K, Ogata E, Yamada-Okabe H. Humanized monoclonal antibody against parathyroid hormone-related protein suppresses osteolytic bone metastasis of human breast cancer cells derived from MDA-MB-231. Anticancer Res 2005;25(6B):3817-23.
16. Zhang J, Dai J, Qi Y, et al. Osteoprotegerin inhibits prostate cancer-induced osteoclastogenesis and prevents prostate tumor growth in the bone. J Clin Invest 2001;107(10):1235-44.
17. Lee HL, Pienta KJ, Kim WJ, Cooper CR. The effect of bone-associated growth factors and cytokines on the growth of prostate cancer cells derived from soft tissue versus bone metastases *in vitro.* Int J Oncol 2003;22(4):921-6.
18. Taichman RS, Cooper C, Keller ET, Pienta KJ, Taichman NS, McCauley LK. Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. Cancer Res 2002;62(6):1832-7.
19. Koutsilieris M. Osteoblastic metastasis in advanced prostate cancer. Anticancer Research 1993; 13: 443-449.
20. Koutsilieris M. Skeletal metastases in advanced prostate cancer: cell biology and therapy. Critical Reviews in Oncology/Hematology 1995; 18: 51-64.
21. Rennebeck G, Martelli M & Kyprianou N. Anoikis and survival connections in the tumor microenvironment: is there a role in prostate cancer metastasis? Cancer Research 2005; 65: 11230-11235.
22. Rosol TJ, Tannehill-Gregg SH, LeRoy BE et al. Animal models of bone metastasis. Cancer 2003; 97: 748-757.
23. Chay CH, Cooper CR, Gendernalik JD et al. A functional thrombin receptor (PAR1) is expressed on bone-derived prostate cancer cell lines. Urology 2002; 60: 760-765.
24. Walz DA & Fenton JW. The role of thrombin in tumor cell metastasis. Invasion & Metastasis 1994; 14: 303-308.

25. Wu JD, Higgins LM, Steinle A et al. Prevalent expression of the immunostimulatory MHC class I chainrelated molecule is counteracted by shedding in prostate cancer. The Journal of Clinical Investigation 2004; 114: 560-568.
26. Blades RA, Keating PJ, McWilliam LJ et al. Loss of HLA class I expression in prostate cancer: implications for immunotherapy. Urology 1995; 46: 681-686
27. Batson OV. The vertebral system of veins as a means for cancer dissemination. Progress in Clinical Cancer 1967; 3: 1-18.
28. Ghossein RA, Scher HI, Gerald WL et al. Detection of circulating tumor cells in patients with localized and metastatic prostatic carcinoma: clinical implications. Journal of Clinical Oncology 1995; 13: 1195-1200.
29. Paget S. The distribution of secondary growths in cancer of the breast. 1889. Cancer Metastasis Reviews 1989; 8: 98-101.
30. Lehr JE & Pienta KJ. Preferential adhesion of prostate cancer cells to a human bone marrow endothelial cell line. Journal of the National Cancer Institute 1998; 90: 118–123.
31. Honn KV & Tang DG. Adhesion molecules and tumor cell interaction with endothelium and subendothelial matrix. Cancer Metastasis Reviews 1992; 11: 353-375.
32. Bogdanos J, Karamanolakis D, Tenta R et al. Endocrine/paracrine/autocrine survival factor activity of bone microenvironment participates in the development of androgen ablation and chemotherapy refractoriness of prostate cancer metastasis in skeleton. Endocrine-Related Cancer 2003; 10: 279-289.
33. Koutsilieris M, Bogdanos J, Milathianakis C et al. Combination therapy using LHRH and somatostatin analogues plus dexamethasone in androgen ablation refractory prostate cancer patients with bone involvement: a bench to bedside approach. Expert Opinion on Investigational Drugs 2006; 15: 795-804.
34. Hsu H, Lacey DL, Dunstan CR et al. Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. Proceedings of the National Academy of Sciences of the United States of America 1999; 96: 3540-3545.

35. Mori K, Le Goff B, Charrier C et al. DU145 human prostate cancer cells express functional receptor activator of NFkappaB: new insights in the prostate cancer bone metastasis process. Bone 2007; 40: 981-990.
36. Fizazi K, Yang J, Peleg S et al. Prostate cancer cells-osteoblast interaction shifts expression of growth/ survival-related genes in prostate cancer and reduces expression of osteoprotegerin in osteoblasts. Clinical Cancer Research 2003; 9: 2587-2597.
37. Koutsilieris M, Frenette G, Lazure C et al. Urokinase-type plasminogen activator: a paracrine factor regulating the bioavailability of IGFs in PA-III cell-induced osteoblastic metastases. Anticancer Research 1993; 13: 481-486.
38. Koutsilieris M, Rabbani SA & Goltzman D. Selective osteoblast mitogens can be extracted from prostatic tissue. The Prostate 1986; 9: 109-115.
39. 45. Mitsiades C, Bogdanos J, Karamanolakis D et al. Bone microenvironment participation in the development of androgen ablation in prostate cancer patients with bone metastases: clinical application of an anti-survival factor therapy. Athens: Paschalidis Medical Publications, 2004, pp. 109-134.
40. Tenta R, Sourla A, Lembessis P et al. Bone microenvironment-related growth factors, zoledronic acid and dexamethasone differentially modulate PTHrP expression in PC-3 prostate cancer cells. Hormone and metabolic research. Hormon- und Stoffwechselforschung. Hormones et metabolisme 2005; 37: 593-601.
41. Mitsiades CS & Koutsilieris M. Molecular biology and cellular physiology of refractoriness to androgen ablation therapy in advanced prostate cancer. Expert Opinion on Investigational Drugs 2001; 10: 1099-1115.
42. Reyes-Moreno C, Frenette G, Boulanger J et al. Mediation of glucocorticoid receptor function by transforming growth factor beta I expression in human PC-3 prostate cancer cells. The Prostate 1995; 26: 260-269.
43. Koutsilieris M & Polychronakos C. Proteinolytic activity against IGF-binding proteins involved in the paracrine interactions between prostate adenocarcinoma cells and osteoblasts. Anticancer Research 1992; 12: 905-910.
44. Srinivas, P. R., Barker, P. & Srivastava, S. Nanotechnology in early detection of cancer. Lab. Invest. 82, 657–662 (2002).

45. Whitesides, G. M. The 'right' size in nanotechnology. Nature Biotechnol. 21, 1161–1165 (2003).
46. Wen Jiang, Betty YS Kim, James T Rutka, Warren CW Chan, Advances and challenges of nanotechnology-based drug delivery systems, Expert Opin. Drug Deliv. (2007) 4(6):621-633.
47. Maynard J, Georgiou G. Antibody engineering. Annu Rev Biomed Eng, 2000;2:339-76.
48. Weiner LM, Adams GP. New approaches to antibody therapy. Oncogene 2000;19:6144-51.
49. Nowakowski GS, Witzig TE. Radioimmunotherapy for B-cell non-Hodgkin lymphoma. Clin Adv Hematol Oncol 2006;4:225-31.
50. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med 2005;353:1659-72.
51. Reck M, Kranich AL, Steinbach AK, et al. EGFR/HER1 inhibition: An example of new targeted therapies in non-small cell lung cancer. Med Klin, Munich 2005;100:785-93.
52. Willett CG, Boucher Y, di Tomaso E, et al. Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. Nat Med 2004;10:145-47.
53. Inoh K, Muramatsu H, Torii S, et al. Doxorubicin-conjugated antimidkine monoclonal antibody as a potential anti-tumor drug. Jpn J Clin Oncol 2006;36:207-11.
54. Vitols KS, Haag-Zeino B, Baer T, et al. Methotrexate-phenylalanine: Optimization of methotrexate prodrug for activation by carboxypeptidase A-monoclonal antibody conjugate. Cancer Res 1995; 55:478-81.
55. Sievers EL, Linenberger M. Mylotarg: Antibody-targeted chemotherapy comes of age. Curr Opin Oncol 2001;13:522-7.
56. Sharkey RM, Goldenberg DM. Targeted therapy of cancer: New prospects for antibodies and immunoconjugates. CA Cancer J Clin 2006;56:226-43.

57. Nielsen UB, Kirpotin DB, Pickering EM, et al. Therapeutic efficacy of anti-ErbB2 immunoliposomes targeted by a phage antibody selected for cellular endocytosis. Biochim Biophys Acta 2002;1591: 109-8.
58. Kirpotin DB, Drummond DC, Shao Y, et al. Antibody targeting of long-circulating lipidic nanoparticles does not increase tumor localization but does increase internalization in animal models. Cancer Res 2006;66:6732-40.
59. Brissette R, Prendergast JK, Goldstein NI. Identification of cancer targets and therapeutics using phage display. Curr Opin Drug Discov Devel 2006;9:363-69.
60. Pasqualini R, Koivunen E, Ruoslahti E. V integrins as receptors for tumor targeting by circulating ligands. Nat Biotechnol 1997;15: 542-6.
61. Beekman KW, Colevas AD, Cooney K, et al. Phase II evaluations of cilengitide in asymptomatic patients with androgen-independent prostate cancer: Scientific rationale and study design. Clin Genitourin Cancer 2006;4:299-302.
62. Bibby DC, Talmadge JE, Dalal MK, et al. Pharmacokinetics and biodistribution of RGD-targeted doxorubicin-loaded nanoparticles in tumor-bearing mice. Int J Pharmacol 2005;293:281-90.
63. Silverman J, Liu Q, Bakker A, et al. Multivalent avimer proteins evolved by exon shuffling of a family of human receptor domains. Nat Biotechnol 2005;23:1556-61.
64. Cortez-Retamozo V, Backmann N, Senter PD, et al. Efficient cancer therapy with a nanobody-based conjugate. Cancer Res 2004;64: 2853-7.
65. Hansson M, Ringdahl J, Robert A, et al. An in vitro selected binding protein (Affibody) shows conformation-dependent recognition of the respiratory syncytial virus (RSV) G protein. Immunotechnology 1999;4:237-52.
66. Gabizon A, Shmeeda H, Horowitz AT, et al. Tumor cell targeting of liposome-entrapped drugs with phospholipid-anchored folic acid- PEG conjugates. Adv Drug Deliv Rev 2004;56:1177-92.
67. Ross JF, Chaudhuri PK, Ratnam M. Differential regulation of folate receptor isoforms in normal and malignant tissues in vivo and in established cell lines. Physiologic and clinical implications. Cancer 1994;73:2432-43.
68. Kamen BA, Capdevila A. Receptor-mediated folate accumulation is regulated by the cellular folate content. Proc Natl Acad Sci USA 1986;83:5983-7.

69. Park EK, Lee SB, Lee YM. Preparation and characterization of methoxy poly(ethylene glycol)/poly(caprolactam) amphiphilic block copolymeric nanospheres for tumor-specific folate-mediated targeting of anticancer drugs. Biomaterials 2005;26:1053-61.
70. Quintana A, Raczka E, Piehler L, et al. Design and function of a dendrimer-based therapeutic nanodevice targeted to tumor cells through the folate receptor. Pharmacol Res 2002;19:1310–6.
71. Gabizon A, Horowitz AT, Goren D, et al. Targeting folate receptor with folate linked to extremities of poly(ethylene glycol)-grafted liposomes: In vitro studies. Bioconjugate Chem 1999;10:289-98.
72. Ohannesian DW, Lotan D, Thomas P, et al. Carcinoembryonic antigen and other glycoconjugates act as ligands for galectin-3 in human colon carcinoma cells. Cancer Res 1995;55:2191-9.
73. Managit C, Kawakami S, Nishikawa M, et al. Targeted and sustained drug delivery using pegylated galactosylated liposomes. Int J Pharmacol 2003;266:77-84.
74. David A, Kopeckova P, Kopecek J, et al. The role of galactose, lactose, and galactose valency in the biorecognition of N-(2-hydroxypropyl) methacrylamide copolymers by human colon adenocarcinoma cells. Pharmacol Res 2002;19:1114-22.
75. Weissleder R, Kelly K, Sun EY, et al. Cell-specific targeting of nanoparticles by multivalent attachment of small molecules. Nat Biotechnol 2005;23:1418-23.
76. Nimjee SM, Rusconi CP, Sullenger BA. Aptamers: An emerging class of therapeutics. Annu Rev Med 2005;56:555-83.
77. Farokhzad OC, Karp JM, Langer R. Nanoparticle-aptamer bioconjugates for cancer targeting. Expert Opin Drug Deliv 2006; 3:311-24.
78. Gopinath SC. Methods developed for SELEX. Anal Bioanal Chem, 2007;387:171-82.
79. Hermann T, Patel DJ. Adaptive recognition by nucleic acid aptamers. Science 2000;287:820-5.
80. Holeman LA, Robinson SL, Szostak JW, et al. Isolation and characterization of fluorophore-binding RNA aptamers. Fold Des 1998; 3:423-31.

81. Tuerk C, Gold L. Systemic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science 1990;249:505-10.
82. Farokhzad OC, Jon S, Khademhosseini A, et al. Nanoparticleaptamer bioconjugates: A new approach for targeting prostate cancer cells. Cancer Res 2004;64:7668-72.
83. Farokhzad OC, Cheng J, Teply BA, et al. Targeted nanoparticleaptamer bioconjugates for cancer chemotherapy in vivo. Proc Natl Acad Sci USA 2006;103:6315-20.
84. Bagalkot V, Farokhzad OC, Langer R, et al. An aptamer-doxorubicin physical conjugate as a novel targeted drug-delivery platform. Angew Chem Int Ed Engl, 2006;45:8149-52.
85. Zhou C, Jiang Y, Hou S, et al. Detection of oncoprotein plateletderived growth factor using a fluorescent signaling complex of an aptamer and TOTO. Anal Bioanal Chem 2006;384:1175-80.
86. Kim ES, Serur A, Huang J, et al. Potent VEGF blockade causes regression of coopted vessels in a model of neuroblastoma. Proc Natl Acad Sci USA 2002;99:11399-404.
87. Shangguan D, Li Y, Tang Z, et al. Aptamers evolved from live cells as effective molecular probes for cancer study. Proc Natl Acad Sci USA 2006;103:11838-43.
88. Chu TC, Marks JW III, Lavery LA, et al. Aptamer toxin conjugates that specifically target prostate tumor cells. Cancer Res 2006;66: 5989-92.
89. McNamara JO II, Andrechek ER, Wang Y, et al. Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. Nat Biotechnol 2006;24:1005-15.
90. C. Kremer, G. Breier,W. Risau, K.H. Plate, Up-regulation of flk-1/vascular endothelial growth factor receptor 2 by its ligand in a cerebral slice culture system, Cancer Res. 57 (17) (1997) 3852–3859.
91. L.F. Brown, B. Berse, R.W. Jackman, K. Tognazzi, E.J. Manseau, D.R. Senger, H.F. Dvorak, Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in adenocarcinomas of the gastrointestinal tract, Cancer Res. 53 (19) (1993) 4727–4735.
92. M.V. Backer, T.I. Gaynutdinov, V. Patel, A.K. Bandyopadhyaya, B.T. Thirumamagal, W. Tjarks, R.F. Barth, K. Claffey, J.M. Backer, Vascular endothelial

growth factor selectively targets boronated dendrimers to tumor vasculature, Mol. Cancer Ther. 4 (9) (2005) 1423–1429.

93. J. Chen, H.Wu, D. Han, C. Xie, Using anti-VEGF McAb and magnetic nanoparticles as double-targeting vector for the radioimmunotherapy of liver cancer, Cancer Lett. 231 (2) (2006) 169–175.
94. L. Li, C.A.Wartchow, S.N. Danthi, Z. Shen, N. Dechene, J. Pease, H.S. Choi, T. Doede, P. Chu, S. Ning, D.Y. Lee, M.D. Bednarski, S.J. Knox, A novel antiangiogenesis therapy using an integrin antagonist or anti-Flk-1 antibody coated 90Y-labeled nanoparticles, Int. J. Radiat. Oncol. Biol. Phys. 58 (4) (2004) 1215–1227.
95. R.E. Nisato, J.C. Tille, A. Jonczyk, S.L. Goodman, M.S. Pepper, alphav beta 3 and alphav beta 5 integrin antagonists inhibit angiogenesis in vitro, Angiogenesis 6 (2) (2003) 105–119.
96. J.D. Hood, M. Bednarski, R. Frausto, S. Guccione, R.A. Reisfeld, R. Xiang, D.A. Cheresh, Tumor regression by targeted gene delivery to the neovasculature, Science 296 (5577) (2002) 2404–2407.
97. N. Nasongkla, X. Shuai, H. Ai, B.D.Weinberg, J. Pink, D.A. Boothman, J. Gao, cRGDfunctionalized polymer micelles for targeted doxorubicin delivery, Angew. Chem., Int. Ed. Engl. 43 (46) (2004) 6323–6327.
98. E. Ruoslahti, Specialization of tumour vasculature, Nat. Rev., Cancer 2 (2) (2002) 83–90.
99. E. Ruoslahti, Fibronectin and its integrin receptors in cancer, Adv. Cancer Res. 76 (1999) 1–20.
100. L. Osborn, C. Hession, R. Tizard, C. Vassallo, S. Luhowskyj, G. Chi-Rosso, R. Lobb, Direct expression cloning of vascular cell adhesion molecule 1, a cytokineinduced endothelial protein that binds to lymphocytes, Cell 59 (6) (1989) 1203–1211.
101. Dienst, A. Grunow, M. Unruh, B. Rabausch, J.E. Nor, J.W. Fries, C. Gottstein, Specific occlusion of murine and human tumor vasculature by VCAM-1-targeted recombinant fusion proteins, J. Natl. Cancer Inst. 97 (10) (2005) 733–747.

102. S. Gosk, T. Moos, C. Gottstein, G. Bendas, VCAM-1 directed immunoliposomes selectively target tumor vasculature in vivo, Biochim. Biophys. Acta 1778 (4) (2008) 854–863.

103. P. Vihinen, R. Ala-aho, V.M. Kahari, Matrix metalloproteinases as therapeutic targets in cancer, Curr. Cancer Drug Targets 5 (3) (2005) 203–220.

104. M. Kondo, T. Asai, Y. Katanasaka, Y. Sadzuka, H. Tsukada, K. Ogino, T. Taki, K. Baba, N. Oku, Anti-neovascular therapy by liposomal drug targeted to membrane type-1 matrix metalloproteinase, Int. J. Cancer 108 (2) (2004) 301–306.

105. L. Genis, B.G. Galvez, P. Gonzalo, A.G. Arroyo, MT1-MMP: universal or particular player in angiogenesis? Cancer Metastasis Rev. 25 (1) (2006) 77–86.

106. K. Atobe, T. Ishida, E. Ishida, K. Hashimoto, H. Kobayashi, J. Yasuda, T. Aoki, K. Obata, H. Kikuchi, H. Akita, T. Asai, H. Harashima, N. Oku, H. Kiwada, In vitro efficacy of a sterically stabilized immunoliposomes targeted to membrane type 1 matrix metalloproteinase (MT1-MMP), Biol. Pharm. Bull. 30 (5) (2007) 972–978.

107. H. Hatakeyama, H. Akita, E. Ishida, K. Hashimoto, H. Kobayashi, T. Aoki, J. Yasuda, K. Obata, H. Kikuchi, T. Ishida, H. Kiwada, H. Harashima, Tumor targeting of doxorubicin by anti-MT1-MMP antibody-modified PEG liposomes, Int. J. Pharm. 342 (1–2) (2007) 194–200.

108. E.I. Deryugina, M.A. Bourdon, K. Jungwirth, J.W. Smith, A.Y. Strongin, Functional activation of integrin alpha V beta 3 in tumor cells expressing membrane-type 1 matrix metalloproteinase, Int. J. Cancer 86 (1) (2000) 15–23.

109. J.J. Laskin, A.B. Sandler, Epidermal growth factor receptor: a promising target in solid tumours, Cancer Treat. Rev. 30 (1) (2004) 1–17.

110. V. Grunwald, M. Hidalgo, Developing inhibitors of the epidermal growth factor receptor for cancer treatment, J. Natl. Cancer Inst. 95 (12) (2003) 851–867.

111. X. Pan, G. Wu, W. Yang, R.F. Barth, W. Tjarks, R.J. Lee, Synthesis of cetuximabimmunoliposomes via a cholesterol-based membrane anchor for targeting of EGFR, Bioconjug. Chem. 18 (1) (2007) 101–108.

112. M. Harries, I. Smith, The development and clinical use of trastuzumab (Herceptin), Endocr. Relat. Cancer 9 (2) (2002) 75–85.

113. L. Nobs, F. Buchegger, R. Gurny, E. Allemann, Biodegradable nanoparticles for direct or two-step tumor immunotargeting, Bioconjug. Chem. 17 (1) (2006) 139–145.

114. D.B. Kirpotin, D.C. Drummond, Y. Shao, M.R. Shalaby, K. Hong, U.B. Nielsen, J.D. Marks, C.C. Benz, J.W. Park, Antibody targeting of long-circulating lipidic nanoparticles does not increase tumor localization but does increase internalization in animal models, Cancer Res. 66 (13) (2006) 6732–6740.

115. M. Singh, Transferrin As A targeting ligand for liposomes and anticancer drugs, Curr. Pharm. Des. 5 (6) (1999) 443–451.

116. N.C. Bellocq, S.H. Pun, G.S. Jensen, M.E. Davis,. Transferrin-containing, cyclodextrin polymer-based particles for tumor-targeted gene delivery, Bioconjug. Chem. 14 (6) (2003) 1122–1132.

117. S. Hu-Lieskovan, J.D. Heidel, D.W. Bartlett, M.E. Davis, T.J. Triche, Sequencespecific knockdown of EWS-FLI1 by targeted, nonviral delivery of small interfering RNA inhibits tumor growth in a murine model of metastatic Ewing's sarcoma, Cancer Res. 65 (19) (2005) 8984–8992.

118. J.D. Heidel, Z. Yu, J.Y. Liu, S.M. Rele, Y. Liang, R.K. Zeidan, D.J. Kornbrust, M.E. Davis, Administration in non-human primates of escalating intravenous doses of targeted nanoparticles containing ribonucleotide reductase subunit M2 siRNA, Proc. Natl. Acad. Sci. U. S. A. 104 (14) (2007) 5715–5721.

119. J. Wu, Y. Lu, A. Lee, X. Pan, X. Yang, X. Zhao, R.J. Lee, Reversal of multidrug resistance by transferrin-conjugated liposomes co-encapsulating doxorubicin and verapamil, J. Pharm. Sci. 10 (3) (2007) 350–357.

120. Moghimi SM, Hunter AC, Murray JC. Long-circulating and target specific nanoparticles: theory to practice. Phar macol Rev 2001;53: 283-318.

121. Zweers MLT, Grijpma DW, Engbers GHM, Feijen J. The preparation of monodisperse biodegradable polyester nanoparticles with a controlled size. J Biomed Mater Res Part B: Appl Biomater 2003;66B:559-66.

122. Labhasetwar V. Nanoparticles for drug delivery. Pharm News 1997;4:28-31.

123. Rouzes C, Gref R, Leonard M, De Sousa Delgado A, Dellacherie E. Surface modification of poly(lactic acid) nanospheres using hydrophobically modified

dextrans as stabilizers in an o/w emulsion/evaporation technique. J Biomed Mater Res 2000;50:557-65.

124. Berton M, Allemann E, Stein CA, Gurny R. Highly loaded nanoparticulate carrier using an hydrophobic antisense oligonucleotide complex. Eur J Pharm Biopharm 1999;9:163-70.

125. D.K. Sahana, et al., PLGA nanoparticles for oral delivery of hydrophobic drugs: influence of organic solvent on nanoparticle formation and release behavior in vitro and in vivo using estradiol as amodel drug, J. Pharm. Sci. 97 (4) (2008) 1530-1542.

126. M.F. Zambaux, et al., Preparation and characterization of protein C-loaded PLA nanoparticles, J. Control. Release 60 (2–3) (1999) 179–188.

127. C. Pinto Reis, et al., Nanoencapsulation I. Methods for preparation of drugloaded polymeric nanoparticles, Nanomedicine 2 (1) (2006) 8–21.

128. J.M. Barichello, et al., Encapsulation of hydrophilic and lipophilic drugs in PLGA nanoparticles by the nanoprecipitation method, Drug Dev. Ind. Pharm. 25 (4) (1999) 471–476.

129. K.S. Soppimath, et al., Biodegradable polymeric nanoparticles as drug delivery devices, J. Control. Release 70 (1–2) (2001) 1–20.

130. K.G. Carrasquillo, et al., Non-aqueous encapsulation of excipient-stabilized spray-freeze dried BSA into poly(lactide-co-glycolide) microspheres results in release of native protein, J. Control. Release 76 (3) (2001) 199-208.

131. M.N. Kumar, et al., Cationic poly(lactide-co-glycolide) nanoparticles as efficient in vivo gene transfection agents, J. Nanosci. Nanotechnol. 4 (8) (2004) 990-994.

132. G. Mittal, et al., Estradiol loaded PLGA nanoparticles for oral administration: effect of polymer molecular weight and copolymer composition on release behavior in vitro and in vivo, J. Control. Release 119 (1) (2007) 77–85.

133. L. Liu, et al., Pectin/poly(lactide-co-glycolide) composite matrices for biomedical applications, Biomaterials 25 (16) (2004) 3201–3210.

134. E.L. Hedberg, et al., In vivo degradation of porous poly(propylene fumarate)/poly(dl-lactic-co-glycolic acid) composite scaffolds, Biomaterials 26 (22) (2005) 4616–4623.

135. S.D. Patil, F. Papadimitrakopoulos, D.J. Burgess, Dexamethasone-loaded poly(lactic-co-glycolic) acid microspheres/poly(vinyl alcohol) hydrogel composite coatings for inflammation control, Diab. Technol. Ther. 6 (6) (2004) 887-897.

136. L. Wang, et al., Preparation, characterization, and in vitro evaluation of physostigmine-loaded poly(ortho ester) and poly(ortho ester)/poly(d,llactide-co-glycolide) blend microspheres fabricated by spray drying, Biomaterials 25 (16) (2004) 3275–3282.

137. R. Di Toro, V. Betti, S. Spampinato, Biocompatibility and integrin-mediated adhesion of human osteoblasts to poly(dl-lactide-co-glycolide) copolymers, Eur. J. Pharm. Sci. 21 (2–3) (2004) 161–169.

138. M.L.a.A.M.L. Hans, Biodegradable nanoparticles for drug delivery and targeting, Curr. Opin. Solid State Mater. Sci. 6 (2002) 319.

139. R.C. Mundargi, et al., Nano/micro technologies for delivering macromolecular therapeutics using poly(d,l-lactide-co-glycolide) and its derivatives, J. Control. Release 125 (3) (2008) 193–209.

140. L. Nobs, et al., Poly(lactic acid) nanoparticles labeled with biologically active neutravidin for active targeting, Eur. J. Pharm. Biopharm. 58 (3) (2004) 483-490.

141. V.P. Torchilin, Multifunctional nanocarriers, Adv. Drug Deliv. Rev. 58 (14) (2006) 1532–1555.

142. S.S. Feng, Nanoparticles of biodegradable polymers for new-concept chemotherapy, Expert Rev. Med. Devices 1 (1) (2004) 115–125.

143. Avnesh Kumari, Sudesh Kumar Yadav, Subhash C. Yadav, Biodegradable polymeric nanoparticles based drug delivery systems, Colloids and Surfaces B: Biointerfaces 75 (2010) 1-18.

144. Kreuter J, Alyautdin RN, Kharkevich DA, Ivanov AA. Passage of peptides through the blood brain barrier with colloidal polymer particles (nanoparticles). *(Brain Res)* 1995, 674(1):171–174.

145. Peracchia MT, Vauthier C, Desmaele D, Gulik A, Dedieu JC, et al. Pegylated nanoparticles from a novel methoxypolyethylene glycol cyanoacrylate-hexadecyl cyanoacrylate amphiphilic copolymer. *(Pharm Res)* 1998, 15(4):550–556.

146. Peracchia MT, Fattal E, Desmaele D, Besnard M, Noel JP, et al. Stealth PEGylated polycyanoacrylate nanoparticles for intravenous administration and splenic targeting. *(J Controlled Release)* 1999a, 60(1):121–128.

147. Peracchia MT, Harnisch S, Pinto-Alphandary H, Gulik A, Dedieu JC, et al. Visualization of in vitro protein-rejecting properties of PEGylated stealth polycyanoacrylate nanoparticles. *(Biomaterials)* 1999b, 20(14):1269–1275.

148. Gref R, Domb A, Quellec P, Blunk T,Muller RH, et al. The controlled intravenous delivery of drugs using PEGcoated sterically stabilized nanospheres. *(Adv Drug Deliv Rev)* 1995, 16(2–3):215–233.

149. Noguchi Y, Wu J, Duncan R, Strohalm J, Ulbrich K, et al. Early phase tumor accumulation of macromolecules: a great difference in clearance rate between tumor and normal tissues. *(Jpn J Cancer Res)* 1998, 89(3):307–314.

150. Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *(J Controlled Release)* 2000, 65(1–2):271–284.

151. Vauthier C, Dubernet C, Fattal E, Pinto-Alphandary H, Couvreur P. Poly(alkylcyanoacrylates) as biodegradable materials for biomedical applications. *(Adv Drug Deliv Rev)* 2003, 55(4):519–548.

152. Brigger I, Dubernet C, Couvreur P. Nanoparticles in cancer therapy and diagnosis. *(Adv Drug Deliv Rev)* 2002, 54(5):631–651.

153. Vauthier C, Dubernet C, Fattal E, Pinto-Alphandary H, Couvreur P. Drug delivery to resistant tumors: the potential of poly(alkyl cyanoacrylate) nanoparticles. *(J Controlled Release)* 2003, 93(2):151–160.

154. Gao K, Jiang X. Influence of particle size on transport of methotrexate across blood brain barrier by polysorbate 80-coated polybutylcyanoacrylate nanoparticles. *(Int J Pharm)* 2006, 310(1–2):213–219.

155. Petri B, Bootz A, Khalansky A, Hekmatara T, Muller R, et al. Chemotherapy of brain tumour using doxorubicin bound to surfactant-coated polybutylcyanoacrylate nanoparticles: revisiting the role of surfactants. *(J Controlled Release)* 2007, 117(1):51–58.

156. Schroeder U, Sommerfeld P, Sabel BA. Efficacy of oral dalargin-loaded nanoparticle delivery across the blood brain barrier. *(Peptides)* 1998, 19(4):777–780.

157. Alyautdin RN, Petrov VE, Langer K, Berthold A, Kharkevich DA, et al. Delivery of loperamide across the blood brain barrier with polysorbate 80-coated polybutylcyanoacrylate nanoparticles. *(Pharm Res)* 1997, 14(3):325–328.

158. Alyautdin RN, Tezikov EB, Ramge P, Kharkevich DA, Begley DJ, et al. Significant entry of tubocurarine into the brain of rats by adsorption to polysorbate 80-coated polybutylcyanoacrylate nanoparticles: an in situ brain perfusion study. *(J Microencapsul)* 1998, 15(1):67–74.

159. Ambruosi A, Gelperina SE, Khalansky AS, Tanski S, Theisen A, et al. Influence of surfactants, polymer and doxorubicin loading on the anti-tumour effect of polybutylcyanoacrylate nanoparticles in a rat glioma model. *(J Microencapsul)* 2006, 23(5):582–592.

160. Ambruosi A, Yamamoto H, Kreuter J. Body distribution of polysorbate 80 and doxorubicin-loaded 14C- polybutylcyanoacrylate nanoparticles after intravenous administration in rats. *(J Drug Target)* 2005, 13(10):535–542.

161. Das D, Lin S. Double-coated poly butylcyanoacrylate nanoparticulate delivery sytems for brain targeting of dalargin via oral administration. *(J Pharm Sci)* 2005, 94(6):1343–1353.

162. Choi YK, Bae YH, Kim SW. Block copolymer nanoparticles of ethylene oxide and isobutyl cyanoacrylate. (Macromolecules) 1995, 28(24):8419–8421.

163. Peracchia MT, Desma¨ele D, Couvreur P, d'Angelo J. Synthesis of a novel poly(MePEG cyanoacrylateco-alkyl cyanoacrylate) amphiphilic copolymer for nanoparticle technology. (Macromolecules) 2003, 30(4:):846–851.

164. Gref R, Luck M, Quellec P, Marchand M, Dellacherie E, et al. 'Stealth' corona-core nanoparticles surface modified by polyethylene glycol (PEG): influences of the corona (PEG chain length and surface density) and of the core composition on phagocytic uptake and plasma protein adsorption. (Biointerfaces) 2000, 18(3–4):301–313.

165. Brigger I, Dubernet C, Couvreur P. Nanoparticles in cancer therapy and diagnosis. (Adv Drug Deliv Rev) 2002, 54(5):631–651.

166. Fleisch HA. Bisphosphonates: Preclinical aspects and use in osteoporosis. *Ann Med.* 1997;29:55–62.

167. Shinoda H, Adamek G, Felix R, Fleisch H, Schenk R, Hagan P. Structure-activity relationships of various bisphosphonates. *Calcif Tissue Int.* 1983;35:87–99.

168. Jung A, Bisaz S, Fleisch H. The binding of pyrophosphate and two diphosphonates by hydroxyapatite crystals. *Calcif Tissue Res.* 1973;11:269–280.

169. Rogers MJ, Gordon S, Benford HL, et al. Cellular and molecular mechanisms of action of bisphosphonates. *Cancer.* 2000;88(12 Suppl):2961–2978.

170. Fleisch H. Bisphosphonates: mechanisms of action. *Endocr Rev* 1998;19:80–100.

171. Goltzman D. Osteolysis and cancer. *J Clin Invest.* 2001;107: 1219–1220.

172. Shipman CM, Rogers MJ, Apperley JF, Russell RGG, Croucher PI. Bisphosphonates induce apoptosis in human myeloma cell lines: a novel anti-tumour activity. *Br J Haematol.* 1997;98:665–672.

173. Shipman CM, Croucher PI, Russell RGG, Helfrich MH, Rogers MJ. The bisphosphonate incadronate (YM175) causes apoptosis of human myeloma cells in vitro by inhibiting the mevalonate pathway. *Cancer Res.* 1998;58:5294–5297.

174. Aparicio A, Gardner A, Tu Y, Savage A, Berenson J, Lichtenstein A. In vitro cytoreductive effects on multiple myeloma cells induced by bisphosphonates. *Leukemia.* 1998;12:220-229.

175. Derenne S, Amiot M, Barille´ S, et al. Zoledronate is a potent inhibitor of myeloma cell growth and secretion of IL-6 and MMP-1 by the tumoral environment. *J Bone Miner Res.* 1999;14:2048–2056.

176. Savage AD, Belson DJ, Vescio RA, Lichtenstein AK, Berenson JR. Pamidronate reduces IL-6 production by bone marrow stroma from muliple myeloma patients. *Blood.* 1996; 88(Suppl 1):105a.

177. Magnetto S, Boissier S, Delmas PD, Cle´zardin P. Additive antitumor activities of taxoids in combination with the bisphosphonate ibandronate against invasion and adhesion of human breast carcinoma cells to bone. *Int J Cancer.* 1999;83:263–269.

178. Boissier S, Ferreras M, Peyruchaud O, et al. Bisphosphonates inhibit breast and prostate carcinoma cell invasion, an early event in the formation of bone metastases. *Cancer Res.* 2000;60:2949–2954.

179. Virtanen SS, Vaananen HK, Harkonen PL, Lakkakorpi PT. Alendronate inhibits invasion of PC-3 prostate cancer cells by affecting the mevalonate pathway. *Cancer Res.* 2002;62: 2708–2714.

180. Teronen O, Konttinen YT, Lindqvist C, et al. Inhibition of metalloproteinase-1 by dichloromethylene bisphosphonate (clodronate). Calcif Tissue Int. 1997;61:59–61.

181. Teronen O, Heikkila¨ P, Konttinen YT, et al. MMP inhibition and down-regulation by bisphosphonates. Ann N Y Acad Sci. 1999;878:453–465.

182. Guise TA, Mundy GR. Cancer and bone. Endocr Rev. 1998; 19:18–54.

183. Guise TA, Yin JJ, Taylor SD, et al. Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis. J Clin Invest. 1996;98:1544–1549.

184. Sahni M, Guenther HL, Fleisch H, Collin P, Martin TJ. Bisphosphonates act on rat bone resorption through the mediation of osteoblasts. J Clin Invest. 1993:91:2004–2011.

185. Wood J, Bonjean K, Ruetz S, et al. Novel anti-angiogenic effects of the bisphosphonate compound zoledronic acid, a potent inhibitor of bone resorption. J Pharmacol Exp Ther. 2002;302:1055–1061.

186. Bonjean K, Bellahcene A, Locigno R, Castronovo V. Zoledronic acid inhibits human endothelial cell adhesion to vitronectin and selectively alters the expression of extracellular matrix cell surface receptors. Bone. 2002;30(3 Suppl): 38S.

187. Boissier S, Colombel M, Delmas P, Cabon F, Cle´zardin P. Zoledronic acid inhibits in vivo experimental angiogenesis. Bone. 2002;30(3 Suppl):38S.

188. Croucher P, De Raeve H, Perry M, et al. Zoledronic acid prevents the development of osteolytic bone disease and increases survival in a murine model of multiple myeloma. Bone. 2002;30(3 Suppl):39S.

189. Hall DG, Stoica G. Effect of the bisphosphonate risedronate on bone metastases in a rat mammary adenocarcinoma model system. J Bone Miner Res. 1994;9:221–230.

190. Pollard M, Luckert PH. Effects of dichloromethylene diphosphonate on the osteolytic and osteoplastic effects of rat prostate adenocarcinoma cells. J Natl Cancer Inst. 1985;75: 949–954.

191. Nemoto R, Uchida K, Tsutsumi M, Koiso K, Satou S, Satou T. A model of localized osteolysis induced by the MBT-2 tumor in mice and its responsiveness to etidronate disodium. J Cancer Res Clin Oncol. 1987;113:539–543.

192. Radl J, Croese JW, Zurcher C, et al. Influence of treatment with APD-bisphosphonate on the bone lesions in the mouse 5T2 multiple myeloma. Cancer. 1985;55:1030–1040.

193. Yaccoby S, Pearse RN, Johnson CL, Barlogie B, Choi Y, Epstein J. Myeloma interacts with the bone microenvironment to induce osteoclastogenesis and is dependent on osteoclast activity. Br J Haematol. 2002;116:278–290.

194. Postlethwaite AE, Land MA, Palmieri GMA, Pifer L, Ingles J. Pamidronate in acquired immune deficiency syndrome. Bone. 1999;24(5 Suppl):32S.

195. Grotz W, Nagel C, Poeschel D, et al. Effect of ibandronate on bone loss and renal function after kidney transplantation. J Am Soc Nephrol. 2001;12:1530–1537.

196. Bissery MC. Preclinical pharmacology of docetaxel. Eur J Cancer 1995; 31A(Suppl 4): S1–6.

197. Ringel I, Horwitz SB. Studies with RP 56976 (Taxotere): a semisynthetic analogue of taxol. J Natl Cancer Inst 1991; 83: 288–291.

198. Bissery MC, Nohynek G, Sanderink GJ, Lavelle F. Docetaxel (Taxotere): a review of preclinical and clinical experience. Part I: Preclinical experience. Anticancer Drugs 1995; 6: 339–368.

199. Crown J, O'Leary M. The taxanes: an update. Lancet 2000; 355: 1176–1178.

200. Shepherd FA, Fossella FV, Lynch T, Armand JP, Rigas JR, Kris MG. Docetaxel (Taxotere) shows survival and quality-of life benefits in the second-line treatment of non-small cell lung cancer: a review of two phase III trials. Semin Oncol 2001; 28(Suppl 2): 4–9.

201. Rowinsky EK, Donehower RC. Antimicrotubule agents. In: Chabner BA, Longo DL (eds.), Cancer chemotherapy and biotherapy. 2nd ed. Lippincott-Raven: Philadelphia, 1996; 263–296.

202. Andreu JM, Diaz JF, Gil R, et al. Solution structure of Taxotere-induced microtubules to 3-nm resolution. The change in protofilament number is linked to the binding of the taxol side chain. J Biol Chem 1994; 269: 31785–31792.

203. Gueritte Voegelein F, Gu_enard D, Lavelle F, Le Goff MT, Mangatal L, Potier P. Relationships between the structure of taxol analogues and their antimitotic activity. J Med Chem 1991; 34: 992–998.

204. Bernard C, Fellous A, Di Leo A, et al. Evaluation of microtubule associated parameters (MTAPs) as predictive markers for advanced breast cancer (ABC) patients treated with docetaxel. Eur J Cancer 2001; 37(Suppl. 6): 182.

205. Wang LG, Liu XM, Kreis W, Budman DR. The effect of antimicrotubule agents on signal transduction pathways of apoptosis: a review. Cancer Chemother Pharmacol 1999; 44: 355–361.

206. Haldar S, Basu A, Croce CM. Bcl-2 is the guardian of microtubule integrity. Cancer Res 1997; 57: 229–233.

207. Griffon-Etienne G, Boucher Y, Brekken C, Suit HD, Jain RK. Taxane-induced apoptosis decompresses blood vessels and lowers interstitial fluid pressure in solid tumors: clinical implications. Cancer Res 1999; 59: 3776–3782.

208. Liekens S, De Clercq E, Neyts J. Angiogenesis: regulators and clinical applications. Biochem Pharmacol 2001; 61: 253–270.

209. Kirsch M, Schackert G, Black PM. Angiogenesis, metastasis, and endogenous inhibition. J Neurooncol 2000; 50: 173–180.

210. Rosen L. Antiangiogenic strategies and agents in clinical trials. Oncologist 2000; 5(Suppl 1): 20–27.

211. Sweeney CJ, Miller KD, Sissons SE, et al. The antiangiogenic property of docetaxel is synergistic with a recombinant humanized monoclonal antibody against vascular endothelial growth factor or 2-methoxyestradiol but antagonized by endothelial growth factors. Cancer Res 2001; 61: 3369–3372.

212. Saaristo A, Karpanen T, Alitalo K. Mechanisms of angiogenesis and their use in the inhibition of tumor growth and metastasis. Oncogene 2000; 19: 6122–6129.

213. Berthold DR, Pond GR, Soban F, De WR, Eisenberger M, Tannock IF. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer: updated survival in the TAX 327 study. J Clin Oncol 2008; 26:242-5.

214. Saad F. Zoledronic acid significantly reduces pathologic fractures in patients with advanced-stage prostate cancer metastatic to bone. Clin Prostate Cancer 2002;1:145-52.

215. Saad F, Gleason DM, Murray R, Tchekmedyian S, Venner P, Lacombe L, et al. Long-term efficacy of zoledronic acid for the prevention of skeletal complications in patients with metastatic hormone-refractory prostate cancer. J Natl Cancer Inst 2004;96:879-82.

216. Oades GM, Senaratne SG, Clarke IA, Kirby RS, Colston KW. Nitrogen containing bisphosphonates induce apoptosis and inhibit the mevalonate pathway, impairing Ras membrane localization in prostate cancer cells. J Urol 2003;170:246-52.

217. B. Karabulut, C. Erten, M.K. Gul, E. Cengiz, B. Karaca, Y. Kucukzeybek, G. Gorumlu, H. Atmaca, S. Uzunoglu, U.A. Sanli, Y. Baran, R. Uslu, Docetaxel/zoledronic acid combination triggers apoptosis synergistically through downregulating antiapoptotic Bcl-2 protein level in hormone-refractory prostate cancer cells, Cell Biology International 33 (2009) 239-246

218. SP Jagdev, RE Coleman, CM Shipman, A Rostami-H and PI Croucher. The bisphosphonate, zoledronic acid, induces apoptosis of breast cancer cells: evidence for synergy with paclitaxel. British Journal of Cancer (2001) 84(8), 1126–1134.

219. Joseph G. Kattan, Fady S. Farhat, Georges Y. Chahine, Fady L. Nasr, Walid T. Moukadem, Fariha C. Younes, Nadine J. Yazbeck , Marwan G. Ghosn. Weekly docetaxel, zoledronic acid and estramustine in hormone-refractory prostate cancer (HRPC), Invest New Drugs (2008) 26:75–79.