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Targeted Nanoparticles for Cancer Therapy

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1. Introduction

The World Health Organization (WHO) estimates that 84 million people will die of cancer between 2005-2015 [1]. According using the WHO mortality database it has been estimated the total number of cancer deaths in the European Union (EU) and in 2012 is predicted to be 1283101, of which 717398 men and 565703 women [2]. The most common types of cancer that will be diagnosed are lung (C33-C34), intestine (colon and rectum; C18-C21) and prostate (C61) for men, and breast (C50), intestine (C18-C21) and lung (C33-C34) for women.

Cancer is a group of diseases which cause an abnormal and uncontrolled cell division coupled with malignant behavior such as invasion and metastasis [3]. A tumor malignant is a neoplasm characterized by a failure in the regulation of tissue growth. The abnormal proliferation of tissues is caused by mutations of genes (oncogenes that promote cell growth and reproduction, and tumor suppressor genes that inhibit cell division and survival). Typically, changes in many genes are required to transform a normal cell into a cancer cell.

It is necessary to improve our knowledge of cancer physiopathology for effective cancer therapy, which will allow discover new anti-cancer drugs and develop novel biomedical technologies. The benefits of traditional chemotherapy are limited by the toxicity associated with anticancer drugs in healthy tissues. The common features of cancer and healthy cells make it difficult to achieve pharmacoselectivity of drugs at the target site.

The development of drug delivery systems that are able to modify the biodistribution, tissue uptake and pharmacokinetics of therapeutic agents is considered the great importance in biomedical research and the pharmaceutical industry. Controlled release in drug delivery can significance enhance the therapeutic effect of a drug. A constant concentration of a drug over an extended period of time keeping the drug concentration within the optimum range,

or a pulsatile drug release in response to an environmental change, can be achieved with controlled drug delivery systems [4]. In these type of systems, the drug is protected from degradation following administration, the delivery system can be administered close to the tumoral cells, the drug is released with a specific patron and the action of the drug on tumoral cells can be direct.

Nanotechnology, refers to the understanding and control of matter at dimensions between approximately 1 and 100 nanometers in at least one dimension. Nanomaterials have a large surface area to volume ratio and their biological and physicochemical properties, such as friction and interaction with other molecules, are distinct from equivalent materials at a larger scale. These new properties open opportunities in a wide variety of areas of technology, ranging from intelligent nanoscale materials to medicine and biology, where first nanotechnology applications have demonstrated an enormous potential [5]. Thus, the term nanomedicine has been taking shape and has been defined as the applications of nanotechnology for treatment, diagnosis, monitoring and control of biological systems by the National Institutes of Health [6]. Nanomedicine attempts to use sophisticated approaches to either kill specific cells or repair them one cell at a time, offering new possibilities towards the development of personalized medicine [7] focused on certain diseases which are currently being investigated, especially cancer.

One of the most important and hopeful tools employed in nanomedicine are nanoparticles (NPs), which are solid, colloidal particles consisting of macromolecular substances that are being developed to: improve drug bioavailability, abrogate treatment-induced drug resistance, and reduce nonspecific toxicity in the field of medicine. Depending on the method of preparation NPs can be constructed to possess different properties and release characteristics for the best delivery or encapsulation of the therapeutic agent [8]. In all these types, drugs can be absorbed onto the surface, entrapped inside, or dissolved within the matrix of the NPs [9]. One advantage of NPs is their ability to overcome various biological barriers and to localize into the target tissue. The first generation of NPs comprises passive delivery systems that, in case of cancer, reach the tumor through the fenestrations in the adjacent neovasculature [10]. The unique mechanism of driving systems to the tumor site is the nanometer size of particles, not specific recognition of the tumor or neovascular targets.

In order to optimize the therapeutic index of antitumor drugs, decreasing their toxicity to normal tissues, a second generation of nanosystems includes additional functionalities that allow for molecular recognition of the target tissue or for active or triggered release of the payload at the disease site. Thus, the presence of reactive pendant groups in NPs make easy their vectorization forward specific cell motif by binding of ligands. These include various ligands [11-13] that bind to specific target cell surface markers or surfacemarkers expressed in the disease microenvironment. Responsive systems, such as pH-sensitive polymers, are also included in this category. Hence, over the past

years, efforts have been focused on the development of nanomedicines such as NPs, liposomes, micelles or dendrimers for the specific delivery of anticancer drugs to tumor tissues [14].

2. Physiological characteristics of solid tumors

Tumors are characterized by poorly differentiated, highly chaotic arrangement of vessel which have endothelial cell-cell junctions and discontinuous basement membrane. Angiogenesis is not only a prerequisite for the transformation from a small, often dormant cluster of cancer cells to a solid tumor, but is also required for the spread of tumor. Microvascular network is absolutely essential for the development of solid tumors. Once a tumor cell cluster, whether in its initial stage as a primary tumor or in later stages when forming metastases, induces an angiogenic switch, its vasculature and microenvironment changes dramatically, and abnormal cellular organization, vessel structure, and physiology function develops (Figure 1). Angiogenesis is defined as the formation of new blood vessels from existing ones. For solid tumors of 1-2 mm^3, oxygen and nutrients can reach the center of the tumor by simple diffusion. Because of their non-functional or non-existent vasculature, non-angiogenic tumors are highly dependent on their microenvironment of oxygen and the supply nutrients. When tumor reaches 2 mm^3, a state of cellular hypoxia begins, initiating angiogenesis.

Angiogenesis is regulated by a fine balance of activators and inhibitors [15]. The vascular endothelial grown factor (VEGF), also called vascular permeability factor (VPF), plays an important role in regulating the process of tumor angiogenesis. VEGF has been shown to stimulate the proliferation, migration and invasion of endothelial by interacting with a family of tyrosine kinase receptor expressed on vascular endothelium. VEGF is also known to have the ability to enhance the permeability of microvessels, favoring the rapid and reversible increases in extravasation of plasma protein in tissue [16]. In the angiogenesis process, different phases can be distinguished: Dilation of existing vessels, endothelial cell activation, migration and proliferation, hyperpermeability of postcapillary venules and vessel destabilization, basement membrane degradation by proteases such as matrix metalloproteases, cathepsines, urokinase and plasmin, endothelial cell migration, vessel formation and angiogenic remodeling [17].

The new tumor vessels formed during angiogenesis differ markedly from those of normal tissues and the neovasculatures is characterized by an irregular shape, high density, and heterogeneity, and also have different oxygenation, perfusion, pH and metabolic states. The abnormal vascular architecture plays a mayor role for an EPR (Enhanced Permeability and Retention) effect [18]. Extensive angiogenesis and hypervasculature, lack of smooth-muscle layer, pericytes, defective vascular architecture: fenestrations, no constant blood flow and direction, inefficient lymphatic drainage that leads to enhanced retention in the interstitium of tumor and slow venous return that leads to accumulation from the interstitium of tumor.

Physiological changes in blood flow within the tumors and in transport properties of tumor vessels are consequences of these vascular abnormalities. The osmotic pressure in tumors is high [19]. The interstitial compartment of tumors is significantly different to that of normal tissues. Primarily, as a result of vessel leakiness and hyperpermeability with a concomitant bulk flow of free fluid into the interstitial space that cannot be removed effectively due to a lack of functional lymphatics, due to cancer cells compress lymphatic vessels causing their collapsed. The lymphatic network transports interstitial fluid and immune cells out of normal tissue and is essential for immune function and maintenance of fluid balance in tissue interstitium. In tumor cells the vessels are compressed by solid stresses. The function of lymphatic vessels depends on their localization, when they are at the periphery of the tumor or the periphery tumor interface possesses functionality, while those within the tumor are functionality defective. VEGF factors (VEGF-C and VEGF-D) and their corresponding receptors have been identified as specific lymphangiogenesis factors in several tumors, and have been implicated in increased lymphatic metastases in numerous tumors [20].
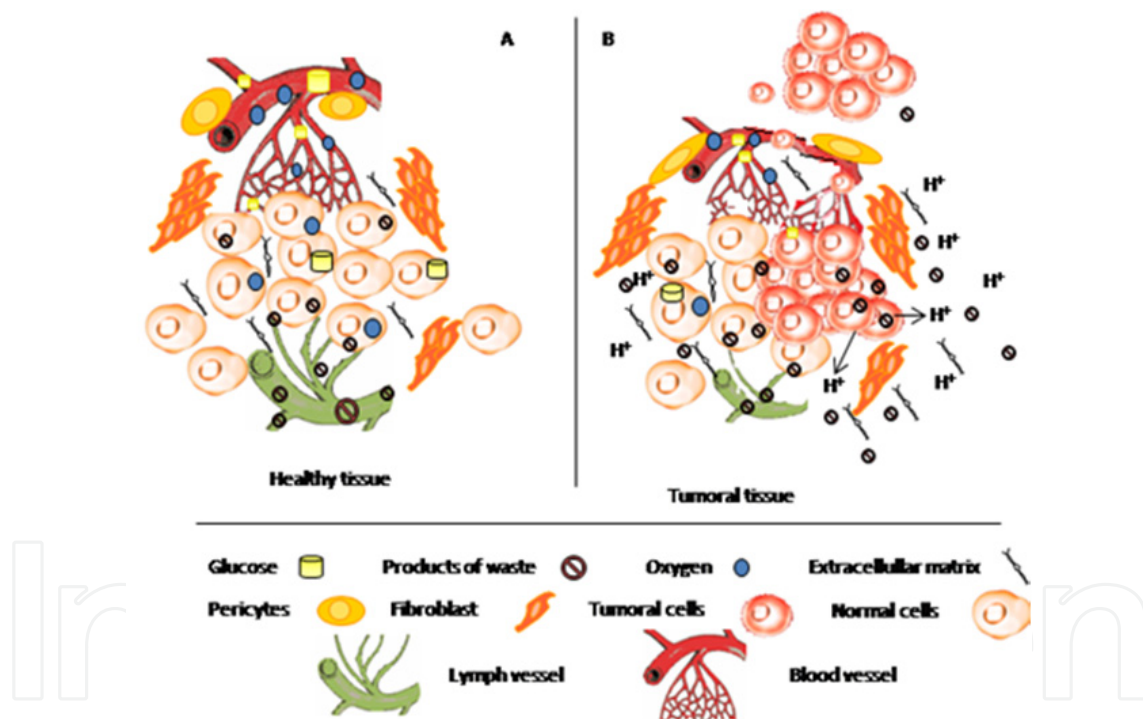
Figure 1. Differences between healthy and tumor tissues. A) Healthy tissue is characterized by a good flow in blood vessel. These vessels are supported by pericytes with a good physiological organization and structure. They provide adequate amounts of glucose and oxygen to normal cells. Collagen fibres, fibroblasts and macrophages are present in the extracellular matrix. Lymph vessels are present and allow the elimination of waste products. B) Tumoral tissue is characterized by vascular disorganization, with fenestrations and discontinuous basement membrane, that promotes the metastasis of abnormal cells to other tissues, inadequate supply of nutrients and poor lymphatic network that does not drain properly increasing the amount of waste products in these tissues and also increasing the protons concentration which decrease the physiological pH. Components of extracellular matrix (collagen fibres, fibroblasts and macrophages) in this type of tumor tissue are also increased

Leaky tumor vasculature and dysfunctional lymphatics in tumor interstitium result in undesirable accumulation of vascular contents in the tumor leading to interstitial hypertension [19]. In normal tissues the interstitial fluid pressure (IFP) is approximately 0 mm Hg, and the pressure in the capillary is around 1-3 mmHg, this gradient facilitates the transport of macromolecules. In tumor tissues the pressure gradient is contrary, consequently, interstitial hypertension results in reduce convection across the walls of tumor blood vessels. IFP tends to be higher at the center of solid tumors, diminishing toward the periphery, creating a mass flow movement of fluid away from the central region of tumor. The microvasculature pressure in tumors is also one to two orders of magnitude higher than in normal tissues.

Abnormal tumor vasculature reduces blood flow and limit delivery of oxygen throughout the tumor resulting in regions of hypoxia. There are different types of hypoxia: inadequate perfusion (ischemia), increased diffusion distance (chronic hypoxia), anemia and hypoxemia [20]. The hypoxic condition initiates signaling events that trigger the upregulation of multiple pro-angiogenic factors in the tumor lesion, another consequence, the lack of oxygen promotes an anaerobic metabolism of tumor cells and an extracellular acidosis in tumor tissues, primarily due to excessive production of lactic acid and CO_2 [20].

So, while the intracellular pH of cells within healthy tissues and tumors is similar, tumors exhibit a lower extracellular pH than normal tissues. Accordingly, although tumor pH may vary according to the tumor area, average extracellular tumor pH is between 6.0 and 7.0, whereas in normal tissues and blood the extracellular pH is around 7.4 [21-22]. Low pH and low pO_2 are intimately linked and a variety of insights now support their roles in the progression of tumor from in situ to invasive cancer [23]. The low extracellular tumor pH mostly arises from the high glycolysis rate in hypoxic cancer cells. However, ATP hydrolysis, glutaminolysis, and ketogenesis also contribute to this extracellular acidic pH.

Therefore, due to the cancer cell presents differences compared to normal cell including vascular abnormalities, interstitial pressure, oxygenation, pH, metabolic states, and abnormal lymphatics, a preferential accumulation of encapsulated drug at desired sites can be obtained either by passive or active targeting.

3. Targeted drug delivery nanoparticles

Targeted NP therapeutics have shown great potential for cancer therapy, as they provide enhanced efficacy and reduced side effects [24]. NP drug delivery can be either an active or passive process. Passive delivery refers to NP transport through leaky tumor capillary fenestrations into the tumor interstitium and cells by passive diffusion or convection [25]. Selective accumulation of NP and drug then occurs by the already mentioned characteristics of the tumor microenvironment (Figure 2).
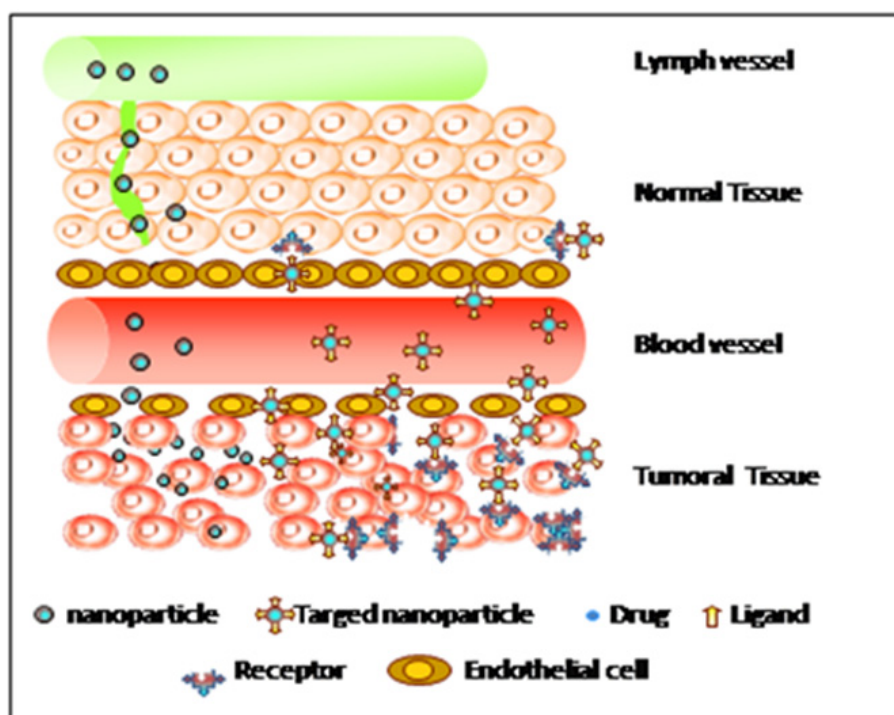
Figure 2. NPs are more able to reach tumor cells through passive targeting due to the characteristics of tumor tissue (vascular disorganization, fenestrations, discontinuous basement membrane, etc.). In normal tissues the lower amount of nanoparticles that able to reach it are removed by lymph vessels while, in tumor tissues, lymphatic network is too damaged to perform its function promoting the accumulation of nanoparticles in the tissue. The functionalized nanoparticles are internalized not only by passive targeting but also by active targeting. This active targeting is more effective in the tumor tissue due to tumor cells overexpress some receptors that allow them a better uptake of functionalized nanoparticles.

Active targeting involves drug delivery to a specific site based on molecular recognition. One such approach is to couple a ligand, such monoclonal antibodies, lectins, aptamers, folate, and peptides, to a NP so that the ligand can interact with its receptor at the target cell site (Figure 2). Depending on the type of ligand-receptor interaction, the rate of cellular internalization would differ. This is an important factor as rates of internalization could affect the accumulation of NP in tumor sites. The use of a targeting moiety also facilitates cellular uptake of the drug by receptor mediated endocytosis, which is an active process requiring a significantly lower concentration gradient across the plasma membrane than simple endocytosis. Thus there is plenty of room to improvise these systems to address the above-mentioned issues and different groups are working to improve the targeting properties of NPs and for the development of targeted therapeutics [26]. In table 1 are shown various nanocarriers evaluated to deliver therapeutic agents into cancer cells.

Ligand	Nanosystem	Drug	Cell/tumor model	Reference
AS1411 DNA apt	nucleolin /liposomes	cisplatin	MCF-7	[27]
	PEG-PLGA NPs	PTX	C6 glioma cells	[28]
Sgc8c apt	SWNTs	Dau	acute lymphoblastic leukemia T-cells (Molt-4).	[29]
Thrombin apt	Mesoporous silica NP	Dtxl	HeLA cells	[30]
EGF	SWNTs	Cisplatin	HNSCC	[31]
antiHER-2 ab: Trastuzamab	PLGA/MMT NPs	PTX	breast cancer	[32]
Tf	PLA-PEG NPs	PTX	BT4C rat glioma model	[33]
	PLGA–NPs	PTX	C6 glioma cells PC3 Prostate cancer cell	[34]
	G4 PAMAM dendrimers	Dox,	C6 glioma cells	[35]
FA	G5 PAMAM dendrimers	MTX	human KB tumor xenografts	[36]
	PEG liposomes	Dox	KB cells	[37]
	NIPA-NPAM-2AAECM	5-FU, TMX	T47D cells, HeLa cells	[38]
cRGD	PEG-PTMC micellar NPs	PTX	U87 MG cells	[39]
Antiαv integrin ab	HSA NPs	Dox	Melanoma cells	[40]

Table 1. Examples of nanocarriers used for active targeted drug delivery

3.1. Aptamers

Originally discovered in 1990, aptamers are short nucleic-acid-based single stranded ligands (DNA, RNA, oligonucleotide), whose size could vary from 20 to 80 nucleotides [27,41], that, through intramolecular interactions, fold into unique tertiary conformations capable of binding to target proteins with high affinity (K_D=10 pmol/l to 10 µmol/l) and specificity. This property makes them an attractive class of targeting molecules as they are also nonimmunogenic and exhibit remarkable stability. Aptamers can tolerate a moderate change in temperature, pH (4-9), and ionic strength, and can be processed with organic solvents without a lost of activity [13]. Aptamers are chemically synthesized and they possess additional advantages over natural antibodies [42] including a smaller size, and single-chain variable fragment antibodies what allows for more efficient penetration into biological compartments [43] and due to which they accumulate quickly within the tumor tissue. It is possible to chemically modify aptamers to facilitate covalent conjugation to nanomaterials, for example, with 50 or 30 amino or thiol groups. These properties in aptamers enable them to withstand the common production conditions encountered during NP preparation. However, due to this small size, aptamers can be cleared quickly by the

kidneys. To delay their clearance, polyethylene glycol (PEG) or cholesterol can be added to aptamer NPs [44-45]. Aptamers that are internalized by cells can be used to study internalization pathways or used as drug targeting agents [24].

Traditionally, a number of compounds were assayed to isolate a ligand for the production of aptamers. However, development of a technique called 'in vitro selection' or Systematic Evolution of Ligands by Exponential Enrichment (SELEX) has allowed the rapid and selective production of aptamers. Briefly, the SELEX method starts with a random library of 1013–1016 single-stranded DNA or RNA and uses an iterative process that specifically amplifies sequences that have high binding affinity to the target molecules [46-47]. Although many complex forms of SELEX exist, there are two basic forms of SELEX (Cell-SELEX and Automated SELEX) [41].

Aptamers can be designed as targeting ligands, and can differentiate diseased cells from healthy cells, thus enabling the selective delivery of therapeutic compounds to target cells [41,47]. A large number of aptamers have been raised against cancer-associated antigens such us AS1411 aptamer for targeting nucleolin protein, which is highly expressed in the membrane of cancer cells [48-49], aptamers CPG 7909 and IMO 2055, that target Toll-like receptor 9 (TLR9), which is expressed by certain immune cells, TD05 aptamer, which was selected for the Burkitt's lymphoma Ramos cell line [47], Sgc8c aptamer which targets leukemia biomarker protein tyrosine kinase-7 (PTK7) [46,50] and can recognize target leukemia cells, DNA aptamers to leukemic lymphoid (CEM) cells [46], and fruoropyrimidine RNA aptamers which target Prostate-specific membrane antigen (PSMA) [51] for targeting prostate cancer. And also another aptamers against antigens such as pigpen [52] for targeting the tumor microvasculature, or mucin 1 (MUC1) [53] for targeting various epithelial neoplasms that upregulate MUC1, whose expression has been associated with carcinomas.

Aptamer-functionalized NPs have also been widely used for cancer cell specific drug delivery. Aptamers that were conjugated to NPs resulted in increased targeting and more efficient therapeutics, as well as more selective diagnostics. For instance, it has been synthesized NPs of poly(D,L-lactide-coglycolyde) [PLGA] and PEG triblock copolymer using aptamers as a targeting ligand for PSMA and Docetaxel (Dtxl)-encapsulated demonstrated that they bind and are taken up by LNCaP prostate ephitelial cells resulting in a significantly enhanced in vitro cellular toxicity as compared with nontargeted NP [24]. In the same way A10 aptamer is being explored for the targeted delivery of several anticancer agents, by including paclitaxel (PTX) and cisplatin in NPs [51,54]. Guo and coworkers conjugated DNA aptamers to a PEG-PLGA NP as a novel drug delivery system capable of targeting cancer cells and endothelia cells in angiogenic blood vessels [28]. In the tested C6 glioma cells, aptamer-nucleolin specific binding resulted in the cellular association of NPs and thereby enhanced the cytotoxicity of the PTX delivery. They suggested the potential of utilizing Ap-PTX-NP as therapeutic drug delivery platform for gliomas treatment [28].

Besides organic NPs, inorganic systems of Au-Ag nanorods (NRs) were synthesized to serve as a platform for binding several aptamer molecules. Thus, Au-Ag NRs have been

conjugated with multiple anti-PTK7 aptamers, such as scg8 aptamer, for targeted cancer photothermal therapy [48,55]. By using Au-Ag NRs that can be conjugated around 80 aptamers, 26 times higher binding affinity was obtained compared to individual aptamer strands [48]. By functionalizing the surface of Au NPs with an RNA aptamer that binds to PSMA, NP–aptamer conjugates were used for targeted molecular computed tomography imaging and treatment of prostate cancer [56].Yin et al. reported a one-step method for the synthesis of DNA-aptamer templated fluorescent silver nanoclusters (AgNCs) [57]. The Sgc8c aptamer strands were immobilized onto AgNCs through cytosine-rich sequence, and the resulting Sgc8c-modified AgNCs showed specific targeting to CCRF-CEM cancer cell over control cells.

In addition to their ability to recognize a target molecule with high specificity, certain aptamers can also modulate the activities of proteins implicated in pathological conditions, making aptamers potentially useful as pharmaceutical agents. For instance, one of the most important success of aptamers so far has been the development of aptamers that are able to bind VEGF [58] such as Pegaptanib sodium aptamer (Macugen, Pfizer, and Eyetech). However, as aptamers are expensive to produce, it is more economical to use aptamers as targeting agents rather than as therapeutic agents. Another example is AS1411, that binds specifically to nucleolin, a bcl-2 mRNA binding protein involved in cell proliferation, which is found on the surface of many cancer cells. Once bound, the AS1411 aptamer is taken into the cancer cell, where it causes death by apoptosis [47,59].

Furthermore, antidotes for anticancer agent toxicities are of interest to regulate drug activity. Thus, aptamers can also be prepared as antidotes for anticancer drugs to modulate anticancer effects. In this way, cDNA aptamer was recently designed for inhibiting cisplatin activity. The multifunctional carrier system consisted of cisplatin as the anticancer agent, which was encapsulated within a liposomal system and conjugated to AS1411-derived aptamer. In the absence of cDNA, the targeted NP showed cell-specific targeting and an improved cytotoxicity. When de cDNA aptamer was administered, it inhibited the cytotoxic activity of cisplatin. However, the interval between the administration of cDNA and NP seemed to be critical [11,27].

3.2. Human epidermal receptor

The Human epidermal receptor (HER)-family tyrosine kinases play a central role in the proliferation, differentiation, and development of cells as they are known to mediate a cell signaling pathway for growth and proliferation in response to the binding of the growth factor ligand [60]. The family consists of four members: epidermal growth factor receptor (EGFR or HER1), HER2 (also known as ERBB2 or HER-2/neu), HER3 and HER4. Each of these receptors has an extracellular region, a single transmembrane region, and a cytoplasmic sequence containing a tyrosine kinase domain and a C-terminal tail [61].

EGFR has six known endogenous ligands: EGF, transforming growth factor-α (TGF-α), amphiregulin, betacellulin, heparin-binding EGF (HB-EGF), and epiregulin [60]. Using any of these ligands as targeting moieties offers a method for targeting the EGFR; especially

TGF-α and EGF as are the most commonly detected in humans. Ligand binding to EGFR results in activation of intracellular signaling cascades in cancer cell proliferation, apoptosis, migration, sensitivity to chemoradiation therapy, and tumor angiogenesis, and the complex is internalized for destruction and recycling [12, 62-63]. Over one-third of all solid tumors have been shown to express EGFR, and in many of these tumors, EGFR expression characterizes a more advanced disease stage [60]. The presence of EGFR corresponds directly to the metastatic capabilities in various types of cancer, such as colorectal [12]. Among the wide range of tumors that overexpress EGFR are breast, lung, colorectal, pancreatic cancers [63], glioblastomas [64], and brain cancers [65].

Hence EGF target delivery systems have been used in cancer molecular imaging diagnosis and therapy [63]. Thus, cisplatin and EGF were attached to single-wall carbon nanotubes (SWNTs) to target squamous cancer cells HNSCC which overexpress EGFR. Through Qdot luminescence and confocal microscopy, it was shown that SWNT–Qdot–EGF bioconjugates was rapidly internalized into the cancer cells, and HNSCC cells were selectively killed in vitro, while tumor growth was regressed in vivo [31]. A current cancer treatment that targets EGFR is the monoclonal antibody Cetuximab, which targets the extracellular domain of EGFR and small-molecule inhibitors of tyrosine kinase activity [66]. One study showed that boronated immunoliposomes with conjugated Fab' fragments of Cetuximab mAb delivered ~8 times more boron to EGFRpositive cells (F98EGFR) than non-targeted IgG immunoliposomes [67].

With regard to HER2, among tumor biomarkers the HER2 membrane receptor is one of the most promising targets for immunotherapy. The surface accessibility, the high level of expression in certain primary and metastatic tumors and the internalization of these antigens via receptor-mediated endocytosis [68] promote preferential intracellular accumulation of drug nanocarriers [69]. The gene encoding HER2 protein is present in normal cells as a single copy and is expressed at low levels in many normal epithelial cells. Amplified HER2 gene and its over-expressed protein product are found in many types of cancers, including breast, ovary, lung, pancreas [63,70], stomach and renal. The overexpression of HER2antigens (*c-erb*B-2, neu) in 20–30% of breast and ovarian cancers is correlated with a high occurrence of metastasis and angiogenesis processes, as well as with a poor prognosis [71]. The ligand binding to the extracellular domain of HERB2 causes the dimerization of the receptor and in this way the activation of many intracellular signaling proteins and physiological pathways, such as the mitogen-activated protein kinases (MAPK) pathway, phospahtidylinositol 3-kinase/AKT/mTOR pathway, and Src tyrosine kinase [61].

Thus, antibodies and antibody fragments, consisting of only the Fab binding regions, against the HER2 receptor are common examples of receptor targets. These antibodies generally exhibit strong interactions with corresponding receptors, with dissociation constants in the nanomolar range. The advantages of the antibody fragments is that they are smaller, and do not contain the Fc region of the antibody which can induce immunogenicity and antigenicity [72]. Antibody-labeled NP is one of the most coveted modes of active targeting of NPs. Blocking the activity of the upregulated receptor by binding it with a ligand, such as

monoclonal antibody (mAb) represented on the nanovector, would ensure arrest of the signalling pathway(s).

Anti-Her2 mAbs (trastuzumab; Herceptin®), a humanized mAb designed to specifically antagonize HER2 function, was approved in 1998 for metastatic breast cancer overexpressing HER2 antigens [70]. Hence Herceptin® is used as a targeting moiety for various NP systems. For instance, incorporation of anti-HER2 antibodies onto the surfaces of PEGylated liposomes has indeed shown greater efficiency for drug delivery compared to non-targeted PEG-liposomes [73] and significantly higher intracellular accumulation was observed with targeted liposomes in xenografts of the HER2 overexpressing BT-474 tumors compared to MCF-7 tumors [69]. It has also been used PTX-loaded anti-HER2 immunonanoparticles (NPs-PTX-HER) which were prepared by the covalent coupling of humanized monoclonal anti-HER2 antibodies (trastuzumab, Herceptin®) to PTX-loaded poly (DL-lactic acid) NPs (NPs-PTX) for the active targeting of tumor cells that overexpress HER2 receptors [71]. NPs-PTX were thiolated and conjugated to activated anti-HER2 mAbs to obtain immunonanoparticles. The immunoreactivity and the *in vitro* efficacy of NPs-PTX-HER were tested on SKOV-3 ovarian cancer cells that overexpress HER2 antigens and it was demonstrated the greater cytotoxic effect of NPs-PTX-HER compared to other PTX formulations. Lyu and coworkers [74] used a single-chain Fv antibody (scFv23) targeting HER-2/neu to deliver tumor necrosis factor (TNF) to TNF-resistant pancreatic cancer cells and compared the cell responses to TNF alone, scFv23/TNF, herceptin, and combinations of scFv23/TNF with various chemotherapeutic agents including 5-Fluorouracil (5-FU), cisplatin, doxorubicin (Dox), gemcitabine, and etoposide. Their results indicated that delivery of TNF to HER2/neu-expressing pancreatic cancer cells using HER2/neu as a targeting molecule may be an effective therapy for pancreatic cancer especially when utilized in combination with 5-FU.

3.3. Transferrin receptor

Transferrin (Tf) (M_w=80 kDa) is the fourth most abundant serum nonheme iron-binding glycoprotein. It is synthesized by the liver and secreted to plasma, where it binds to endogeneous iron, forming the iron-transferrin chelate which is an important physiological source of iron for cells in the body. It helps to transport iron to proliferating cells [75], which is required as a cofactor for DNA synthesis [76], and it also plays a pivotal role in the transportation of iron for the synthesis of hemoglobin. Based on these facts, Tf can be potentially utilized as a cell marker for tumor detection.

Normally at a cell, Tf offloads the iron onto a transferrin receptor (TfR). The natural ligand for TfR, Tf, binds to its receptor with a dissociation constant of around 40nM. TfR, also known as CD71, is a dimeric transmembrane glycoprotein (180 kDa) [77]. The receptor for Tf, referred to as TfR1, is ubiquitously expressed at low levels in most normal human tissues. A second member of the TfR family is TfR2, a protein that is homologous to TfR1 but whose expression is largely restricted to hepatocytes [76]. This receptor is an attractive molecule for the targeted therapy of cancer since it is upregulated on the surface of many

cancer types and is efficiently internalized. Serving as the main port of entry for iron bound Tf into cells, TfR1 is a type-II receptor that resides on the cell membrane and cycles into acidic endosomes into the cell in a clathrin/dynamin dependent manner [78]. The low pH environment triggers dissociation of the iron and the iron-poor Tf is released out of the cell for recycling. As cancer cells rapidly proliferate, the TfR is overexpressed in the surface of malignant cells due to the increased requirement of iron [12]. In this sense, many studies have indicated that the expression level of TfR on tumor cells is much higher than that on normal cells [79], such as the surface of cerebral endothelium and brain tumor cells [80], breast cancer, prostate cancer, and squamous cell carcinomas [63]. This enhanced TfR expression, at levels correlating with the grade of malignancy [81], can be exploited for actively delivering anticancer agents specifically to tumor tissues. This receptor can be targeted in two ways: 1) for the delivery of therapeutic molecules into malignant cells or 2) to block the natural function of the receptor leading directly to cancer cell death [78].

A wide variety of therapeutic agents have been used for TfR-targeted cancer therapy. They include chemotherapeutic drugs, bacterial toxins, plant toxins, DNA, oligonucleotides, short inhibitory RNA (siRNA), and enzymes. Vast types of anti-cancer drugs that have been delivered into cancer cells employing a variety of receptor binding molecules including the use of its natural ligand Tf, anti-TfR antibodies, or TfR-binding peptides alone or in combination with carrier molecules including NPs and viruses [78].

With regard to NPs, Tf has a number of properties that allow it to be successfully incorporated as a targeting ligand in NP systems, such as its stability over a wide pH range (3.5-11) and that has shown to be unaffected by repeated freeze-thaw cycles; hence, it can be subjected to processing conditions commonly encountered during NP preparation [11]. Furthermore, Tf is available in recombinant version (Optiferrin) [82] and, as a human protein, has low immunogenicity [83]. Normally, Tf can be conjugated to NPs less than 100 nm in size to obtain an enhanced cytotoxic activity. If the NPs are greater than 100 nm, it may lead to poor accumulation of these NPs in the tumor cells, which results in moderate anticancer activity. To overcome this issue, the actively targeted system can be directly administered into the tumor tissue by intratumoral injection [84].

Tf-conjugated NPs have been explored in a number of studies for the delivery of anticancer agents. Thus, gold NPs were conjugated with Tf molecules for targeting, imaging and therapy of breast cancer cells (Hs578T, ATCC), showing that, the Tf–TfR-mediated cellular uptake of gold NPs is six times of that in the absence of this interaction [85].

It has also been [86] prepared PTX loaded NPs with shells formed of the biodegradable polymer, PLGA, conjugated to Tf via epoxy linkages. The Tf-conjugated NPs demonstrated greater cellular uptake and reduced exocytosis, yielding greater antiproliferative activity and more sustained effects compared to the free drug or unconjugated NPs. In a similar way, particulate nanodrugs consisting of PLGA loaded with PTX were conjugated to Tf (PTX–NPs–Tf) using an epoxy compound (Denacol-EX-521) [84]. These PTX–NPs–Tf showed a 70% in vitro inhibition of proliferation in human prostate cancer PC3 cells, while at the same concentration the NPs without ligand exhibited 25% inhibition, and PTX in

solution resulted in a 35% [84]. Tf-conjugated lipid-coated PLGA NPs carrying the aromatase inhibitor, 7α-($4'$-amino)phenylthio-1,4-androstadiene-3,17-dione (7α-APTADD), were synthesized and evaluated for aromatase inhibition efficiency in SKBR-3 breast cancer cells. PLGA NPs loaded with the 7α-APTADD were significantly more effective preventing proliferation of the human breast cancer cell line SK-BR-3 than non-targeted NPs. These results suggested that the aromatase inhibition activity of the Tf-NPs was enhanced relative to that of the non-targeted NPs, which was attributable to TfR mediated uptake [87]

Gan and coworkers observed that Tf-conjugated poly(lactide)-D-α-tocopheryl polyethylene glycol succinate diblock copolymer NPs loaded with Dtxl could be more efficient eliciting cytotoxicity against C6 glioma cells than other nontargeted formulations [88].

Transferrin–PEG–adamantane (Tf-PEG-AD) conjugates synthesized for NP modification have been used to target malignant tumors including Ewing's sarcoma [89-90]. Thus, several Tf-NPs have been successfully entered into clinical trials. CALA-01 [91] is one of the first clinically successful transferrin-conjugated nanoparticulate system. This system consists of a duplex of synthetic nonchemically modified siRNA, which self-assembles to a cationic copolymer containing cyclodextrin, AD-PEG as a stabilizing agent, and AD-PEG-Tf as the targeting moiety. After administration, the nanocomplex provides siRNA protection from nucleases in the serum, minimizes erythrocyte aggregation, and reduces complement fixation. At the tumor site, the Tf binds to the tumor cell TfR, which leads to preferential uptake of the complex within the tumor cell. In the cell, the polymer unpacks from the small interfering RNA allowing it to interfere with RNA resulting in reduced tumor growth [92].

Hydroxycamptothecin (HCPT)-loaded stealth niosomes(NS) modified with transferrin (Tf-PEG-NS) were prepared with poly(methoxy-polyethylene glycol cyanoacrylate-co-hexadecyl cyanoacrylate) (MePEG-PHDCA) as surface modification material [93]. Tf-PEG-NS demonstrated the strongest cytotoxicity to three carcinomatous cell lines (KB, K562 and S180 cells), the greatest intracellular uptake especially in nuclei, the highest drug concentration and largest area under the intratumoral HCPT concentration curve, as well as the most powerful anti-tumor activity compared with other niosomes. More reciently Tf modified stealth NPs (Tf-PEG-NP) encapsulating PEG-HCPT conjugate were prepared and was studied the possibility of combination of the functions of passive and active targeting by Tf-PEG-NP, as well as sustained drug release in tumor by PEGylated drug for most efficient tumor targeting and anti-tumor effects enhancement. The advantages of such system included prolonging drug residence time in circulation and increasing EPR effect by the sterically stabilising action of PEG-PHDCA NPs, active targeting function of transferrin by transferring receptor-mediated endocytosis, and sustained releasing drug in tumor by PEGylation of the drug. The prepared Tf-PEG-NP showed more sustained in vitro release profile. The pharmacokinetic and biodistribution studies found that Tf-PEG-NP demonstrated the longest retention time in blood, the highest tumor accumulation, as well as the most powerful anti-tumor activity with the inhibition rate up to 93% against S180 tumor in mice [94].

A pH-sensitive dual-targeting drug carrier (G4-Dox-PEG-Tf-TMX) was synthesized with Tf conjugated on the exterior and Tamoxifen (TMX) in the interior of the fourth generation (G4) Poly(amido amine))(PAMAM) dendrimers for enhancing the blood brain barrier (BBB) [35].

The pH-triggered Dox release was 32% at pH 4.5 and 6% at pH 7.4, indicating a comparatively fast drug release at weak acidic condition and stable state of the carrier at physiological environment. MDR proteins, such as P-glycoprotein (P-gp), MRP4 (ABCC4), and breast cancer resistance protein (BCRP), are over expressed on the BBB and glioma cells, thus causing the block of overcoming the BBB and low uptake of drugs by the tumor cells [95]. The in vitro assay of the drug transport across the BBB model showed that G4-Dox-PEG-Tf-TMX exhibited higher BBB transportation ability. The carrier was internalized into C6 glioma cells upon crossing the BBB model by the coactions of TfR-mediated endocytosis and the inhibition effect of TMX to the drug efflux transports. Moreover, it also displayed the in vitro accumulation of DOX in the avascular C6 glioma spheroids made the tumor volume effectively reduced.

But, besides of its natural ligand, Tf, it have also been used other ligands conjugated to NPs. Thus, antibodies and antibody fragments against the TfR are common examples of receptor targets [72]. Among these are the mAbs A24 [96], Rat anti-murine TfR RI7 217 and YE1/9.9, Murine anti-human TfR Antibody HB21(also known as 5E9), Antibody 454A12, Antibody B3/25, Antibody OKT9, R17217 and OX26 mAb [95,97-99].

For instance the R17217, a rat IgG2a antibody against the mouse TfR which binds to this receptor on mouse cells [99], and the OX26, a murine Ab to the rat TfR, which is used for the delivery of peptides across the BBB [100], have been used in NP systems. Hence, it has been developed human serum albumin (HSA) NPs to which Tf was coupled, and was evaluated the potential of these NPs to deliver drugs across the BBB and, in addition, the possibility of achieving similar results by the coupling of the above-mentioned mAbs against the TfR receptor to the NPs was investigated. The analgesic Loperamide was chosen as the model drug since it does not cross the BBB [101]. HSA NPs coupled to Tf or TfR-mAb are enabling a significant loperamide transport across the BBB into the brain. The loperamide-loaded, Tf- or TfR-mAb-coupled HSA NPs achieved strong antinociceptive effects, whereas IgG2a-modified HSA NPs were not able to transport this drug across the BBB [80]. Therefore, these novel NPs with attached Tf or TfR-mAb represent very useful carriers for the transport of drugs into the brain. It have also been used fluorescein labeled Chitosan (CS) nanospheres conjugated with PEG obtained with the PRINT (Particle Replication In Non-wetting Templates) technology that were bioconjugated either with the OKT9 murine anti-human TfR antibody (NPs–OKT9) or with human Tf (NPs–hTf) [102]. In both cases greater than 80% uptake was observed in several human tumor cell lines (HeLa, Ramos, H460, SKOV-3, HepG2, and LNCaP) compared to bovine Tf conjugated NPs (NPs–bTf) or control IgG1 (NPs–IgG1). The targeting efficiency was dependent on nanocarrier concentration, ligand density, dosing time, and level of cell surface receptor expression. For these cells a strong correlation was found between the viability and the amount of ligand (OKT-9 or hTf) that can be conjugated to the surface of the NPs, with lower cell viability associated with higher percentage of ligand conjugate, suggesting that the polyvalency of the moiety targeting TfR plays a role in the toxicity in some malignancies [102].

3.4. Folate receptor

Folic acid (FA) or folate, a member of the B complex group of vitamins with small-molecular weight (441 Da), is required by eukaryotic cells as is an important co-factor in one-carbon

transfer reactions for biosynthesis of nucleotide bases (purines and pyrimidines) and plays a key role in DNA and RNA synthesis, epigenetic processes, cellular proliferation, and survival [103-104]. Since folic acid is required for essential cell function, the cargo attached the ligand is retained within an endocytic vesicle or released into the cytoplasm. FA conjugates have the ability to deliver a variety of drugs or imaging agents to pathological cells without causing harm to normal tissues. Furthermore FA targeting is an interesting approach for cancer therapy because it offers several advantages over the use of monoclonal antibodies. Thus, FA is known to be stable, inexpensive, non-toxic, non-immunogenic, easy to conjugate to carriers [105], and FA-conjugated drugs or NPs are rapidly internalized via receptor-mediated endocytosis.

Distinct transporters mediate cellular FA uptake. Among them, the FA transporter named as the folate receptor (FR) [9]. Three FR isoforms (FR-α, FR-β and FR-γ) have been identified in human tissues and tumors. FA can be internalized in cells by a low-affinity (K_D of approximately 1-5 μmol/l) membrane-spanning protein, which transports reduced FAs directly into the cytosol or it can be endocytosed by a high-affinity glycoprotein (K_D of approximately 100 pmol/l). FR, often referred to as the high affinity folate-binding protein, is a 38 kDa cell surface glycosyl-phophatidylinositol (GPI)-anchored glycopeptides that characteristically binds folic acid and transports it by a nonclassical endocytic mechanism [106]. The receptor-mediated uptake of FA conjugates proceeds through a series of distinct steps [107]. The process begins with the conjugate binding to FRs on the cell surface. The plasma membrane then invaginates and eventually forms a distinct intracellular compartment. The endocytic vesicles become acidified, and then lysozymes are activated allowing the FR to release the FA conjugates. The membrane-bound FRs recycle back to the cell surface, allowing them to mediate the delivery of additional FA conjugates. Concurrently, the FA conjugates released from FRs escape the endosome, resulting in drug deposition in the cytoplasm. Functional FRs are largely localized to the apical surfaces of polarized epithelia [105]. Normal tissues express insignificant level of FR-α and low level of FR-β (such as liver), and FR-γ is only found in haematopoietic cells. However, FR-α and FR-β are vastly overexpressed in many human tumors such as uterus, colon, lung, prostate, ovaries, mammary glands, nose, throat and brain [11,107-108] which makes it a rational target for drug delivery to tumor tissues. At the tumor site, FA has a very high affinity for tumor cell surface FR and the complex is rapidly internalized into tumor cells (3×10^5 FA molecules/h) [109]. Studies have shown a significant correlation between FR-α expression and the grade and differentiation status of the tumor, thus poorly differentiated and aggressive tumors express high levels of FR-α [110]. However, immunochemistry studies have shown the overexpression of FA receptors in normal tissues like placenta and kidneys [13].

A wide range of chemical conjugates of FA, antifolate drugs, and immunological agents have been used for developing therapeutic and imaging agents for various diseases. Thus, it is not surprising that FA targeted NPs have shown to be effective in a number of tumors. A range of polymers with an improved biocompatibility have been used for the development of FR-targeted NPs [11]. In a typical FR-targeted NP, the anticancer agent is encapsulated in a stabilizing polymer and the FA is conjugated on the surface of the polymer. PEG is often used as a polymer in a FR-targeted nanoparticulate system to enhance its circulation time

and also to improve the association of the targeted NP with the tumor cells [111]. The surface density and length of PEG chains should be optimal to maintain the system targeting and stealth properties [72]. The mole fraction of FA added to a NP system is also thought to affect the cytotoxic capability of the system. It is presumed that higher ligand content would give an enhanced targeting ability. However, when excessive FA molecules are present on the surface of the NPs, they can self-assemble to form dimers, trimers or tubular quartets, which cannot interact with FR (only one molecule of FA can bind to FR) [112]. FA-PEG-liposome loaded with Dox showed a 45-fold higher uptake in FR-rich KB cells compared to nontargeted liposomal-doxorubicin and 86-fold greater cytotoxicity. In mice bearing KB cell tumor xenografts, treatment with FA-targeted liposomal Dox produced a 31% inhibition of tumor growth [37].

Similar to PEG, PLGA NPs can be coated with FA to target the FR to further enhance accumulation of these NPs into tumor cells [113]. Copolymeric nanohydrogels based on N-isopropylacrylamide (NIPA), N-(pyridin-4-ylmethyl)acrylamide (NPAM) and tert-butyl-2-acrylamidoethyl carbamate (2AAECM), as well as FA-conjugate copolymeric nanogels, were synthesized and evaluated for antitumor therapy by loading them with TMX and 5-FU. Nanohydrogels were assayed as TMX and 5-FU delivery systems in vitro. Cell culture experiments were performed using MCF7, T47D and HeLa cells which have different degrees of FR expression. FA-targeted nanohydrogels showed a larger uptake into T47D and HeLa cells due to the fact that these cells are FR-positive. Furthermore, TMX-loaded and 5-FU-loaded nanohydrogels showed effective elimination of carcinoma cells [38]. Loaded with the same drugs, it have also been synthesized FA-conjugate poly[(p-nitrophenyl acrylate)-co-(N-isopropylacrylamide)] systems. TMX and 5-FU-loaded folate-systems present effective elimination of both MCF7 and HeLa cellular lines, and the presence of folate in the particles enhances their internalization, especially in HeLa cells [114-115].

A natural polymer (poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), PHBHHX) was used as a base matrix for the production of a novel nanotherapeutic including antineoplastic agent, etoposide and attached FA as a ligand on the NPs. In the cytotoxicity tests, etoposide loaded and folic acid attached PHBHHX NPs were observed as more effective on HeLa cells than etoposide loaded PHBHHX NPs without attached folic acid. Furthermore the cytotoxicity of folic acid conjugated PHBHHX NPs to cancer cells was found to be much higher than that of normal fibroblast cells, demonstrating that the FA conjugated NPs has the ability to selectively target to cancer cells [116].

CS NPs have also been conjugated to FA to target contrast dye to tumor tissues. The mucoadhesive property of CS provides sustained interaction with the target cells and the FR-mediated uptake leads to an enhanced imaging effect [117]. The cytotoxic activity of CS NP conjugated to FA has also been explored to show a higher cellular cytotoxicity due to enhanced uptake by receptor-mediated endocytosis complemented with a depot effect, which leads to sustained drug release providing grater apoptosis and enhanced cell cycle arrest [118]. An alginate-complexed FA CS NP has been reported for photodynamic early detection of colorectal carcinoma. These NPs are readily engulfed by the cancer cells through FR-mediated endocytosis, furthermore an improved release in the cellular lysosome was observed when they are loaded with 5-aminolevulinic acid (5-ALA) [119].

In other system, FA was coupled with HSA NPs through carbodiimide reaction resulting in the formation of HSA-NPs spheres. The cellular binding and uptake was studied in normal foreskin fibroblasts (HFF), human neuroblastoma cells UKF-NB3, and in rat glioblastoma cell lines. An inceased NP uptake was observed in cancer cells, but not in normal HFFs [9].

3.5. Integrin

Integrins are heterodimeric cell-surface receptors that consist of α- and β-subunits, such as integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, and which are barely detectable or entirely absent from normal blood vessels but are abundantly expressed on tumor-associated endothelial cells [120-121]. Furthermore the $\alpha_v\beta_3$ integrin is important in the calcium-dependent signaling pathway leading to endothelial cell migration [122]. 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 $\alpha_v\beta_3$ integrin [122]. Thus, integrins represent potential pharmacological targets for antiangiogenic therapy. Several antibodies and peptides capable of functionally blocking the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins have been demonstrated to inhibit neovascularization in tumor-bearing mice. The targeting scheme for the $\alpha_v\beta_3$ integrin has centered upon the three amino acid sequence RGD. An important characteristic of the $\alpha_v\beta_3$ integrin is that it is intrinsically associated with VEGFR-2 signaling. Upon $\alpha_v\beta_3$ integrin binding to the components that harbor the RGD sequence, there is an upregulation of VEGF signaling in cell cultures. By blocking $\alpha_v\beta_3$ integrin binding, there would be a reduction in VEGF signaling, proving the use of $\alpha_v\beta_3$ blocking agents for anti-angiogenesis [120]. Targeting the $\alpha_v\beta_3$ integrin with an active targeting NP system increases the effectiveness of anti-angiogenic treatments by the downregulation of VEGF.

Park and coworkers [123] reported the development of self-assembled hydrogel NPs capable of imbibing a peptide sequence that specifically binds to $\alpha_v\beta_3$ integrin. The authors observed that NPs made of hydrophobically modified CS could release the peptide in a sustained manner, and showed that they might be useful for monitoring or destroying angiogenic vessels. Peptides that contain RGD domains can preferentially bind cells in tumor microvasculature that express the $\alpha_v\beta_3$ integrin [13]. However, RGD sequences also act as adhesive molecules and can non-specifically bind tissues that also express its integrin complement. Integrin receptors are also expressed on the cell membrane of macrophages [124] and it is shown that RGD bioconjugates aggregate in spleen and liver tissues due to macrophage clearance [125].

Using an RGD-targeted stealth system, NPs carrying Dox were found to accumulate faster and in higher concentrations in the liver and the spleen [126]. The ligands are incorporated as RGD-PEG-lipid conjugates, which indicates their extension from the NP surface as a consequence of the brush-like state. A report showed that short peptide-targeted NPs exhibited lower cell-bindings abilities when higher mol% of PEG2000 was included into the formulation [127-128]. As a sufficient PEG coating is essential for avoiding recognition by the RES, ligands should be extended away from NP surfaces, to avoid shielding by the

polymer chains. Another study reported the targeting and imaging of MDA-MB-231 human breast cancer cells using RGD peptide-labeled Fluorescent silica NPs (FSiNPs). The FSiNPs exhibited high target binding to $\alpha_v\beta_3$ integrin receptor (ABIR)-positive MDA-MB-231 breast cancer cells in vitro [129].

Peptide-labeled NPs may also be used for targeted gene silencing. A study shows that RGD-CS-NP is a highly selective delivery system for siRNA with the potential for broad applications in human disease [130]. Binding of RGD-CS-NP with $\alpha_v\beta_3$ integrin and antitumor efficacy were examined, resulted in significant inhibition of tumor growth compared with controls. The targeted RGD non-peptide mimetic coupled to NPs were coupled to cDNA encoding ATPμ-Raf tagged with the FLAG epitope [131] and were proven to cause tumor regression in M21-L melanomas. Peptides harboring RGD sequences have also shown high efficiencies in targeting SLK tumor endothelial cells derived from Kaposi's sarcoma. A cyclic RGD pentapeptide was conjugated to the surface of Dox-loaded micelles at different densities. 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 Dox-loaded micelles as compared to the non-targeted micelles [132].

There are studies with other ligands. Thus, integrin-targeted C16Y peptide-modified liposomes (C16Y-L) were prepared to enhance the intracellular uptake of drugs and genes specifically into tumor tissues [133]. The C16Y peptide is a 12-amino acid modified C16 synthetic peptide (DFKLFAVYIKYR-GGC), which is derived from the laminin γ1 chain, and binds to integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ [134]. The cellular uptake of C16Y-L by both endothelial cells and cancer cells was higher than uptake of the un-labeled and scramble peptide-modified liposomes. Moreover, to evaluate whether the uptake depended on an integrin–ligand interaction, they examined the inhibition of C16Y-L uptake using recombinant integrin $\alpha_v\beta_3$ and found that the cellular uptake of C16Y-L treated with $\alpha_v\beta_3$ integrin decreased. This result suggests that C16Y-L can selectively target cells that highly express integrin $\alpha_v\beta_3$.

Finally, a new strategy is to use a multi-targeting NP systems. For instance, PTX-loaded NPs based on Herein, an hyperbranched amphiphilic poly[(amine-ester)-co-(D,L-lactide)]/1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine copolymer (HPAE-co-PLA/DPPE), which was modified with two targeting ligands, RGD and Tf were synthesized [135]. Thus, these dual-targeting NPs may achieve more accumulation and improved lethality of the PTX-loaded NPs in tumors. Active tumors targeting can be achieved in two steps: the ligand RGD enhances the targeting migration and accumulation of NPs to the $\alpha_v\beta_3$ integrin-expressing tumor vasculature and Tf then improves the cellular uptake of NPs by TfR-expressing tumor cells. In addition, a heterobifunctional cross-linker, p-maleimidophenyl isocyanate (PMPI), used for hydroxyl to sulfhydryl coupling was introduced to the HPAE-co-PLA/DPPE copolymer for the successful modification of targeting ligands [136-137]. Results showed the cytotoxicity and cellular uptake of PTX-loaded NPs against human cervical carcinoma (HeLa) cells for their tumor-targeting effects [135].

4. Tumor cell targeting

Cancer cells express different targets on their surface, some of them specific of each type of cancer. Active targeting of nanosystems for cancer treatment has been usually associated with a type of cancer and so with a specific target.

While chemotherapy has been the standard of care for patients with different types of cancer, efforts have shifted toward evaluating novel targeted agents in an attempt to improve outcome. These targeted agents are directed towards key components in several signaling pathways. The potential of targeted therapies has stimulated the study of targeted nanocarriers that can allow synergistically act by binding and inhibiting cancer pathways while delivering therapeutic payloads. Tumor cell targeting involves many targets associated with the uncontrolled cell proliferation and the angiogenesis and others specifics for the different types of cancer (Table 2)

CANCER TYPE	TARGET	AGENT	REFERENCE
Lung cancer (NSCLC)	VEGFR	Axitinib (Pfizer Inc., USA)	[138]
		Cediranib (Recentin®, AstraZeneca plc, UK)	[139]
	EGFR	Cetuximab (Erbiux®, ImClone/Bristol-Myer Squibb, USA)	[140]
		Erlotinib (Tarceva®, Genentech/Roche, Switzerland)	[141]
	IGF-1R	Figitumumab (CP-751871, Pfizer, USA)	[142]
Colorectal cancer	VEGF-A	Bevacizumab (Avastin®, Roche-Pharma AG, Germany)	[143-145]
	EGFR	Cetuximab (Erbiux®, ImClone/Bristol-Myer Squibb, USA)	[146]
		Panitumumab (Amgen Inc; Thousand Oaks, USA)	[147]
Breast cancer	HER2	Transtuzumab (Herceptin®, Genentech)	[148]
		Transtuzumab-DM1 (T-DM1; Genetech Inc/Roche)	[149]
		Pertuzumab (Omnitarg®; Genentech/Roche)	[150]
	PARP	Olaparib (AZD2281; AstraZeneca)	[151]
Prostate cancer	17-α-hydroxylase	Abiraterone acetate (Zytiga, Cougar Biotechnology)	[152]
	AR	MDV3100	[153]
	EGFR	Cetuximab (Erbiux®, ImClone/Bristol-Myer Squibb, USA)	[154]
	HER2	Transtuzumab (Herceptin®, Genentech)	[155]
	HER3	MM-121 (humanized antibody)	[156]
	PSMA	J591 (monoclonal antibody)	[157]

Table 2. Examples of targets for different types of cancer

4.1. Lung cancer

Non-small cell lung cancer (NSCLC) involves signaling pathways that influence angiogenesis, tumorigenesis and tumor growth, and different targeted agents have been used towards vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), EGFR and insulin-like growth factor 1 receptor (IGF-1R) [158-159]. Furthermore, there is an increasing interest in using combinations of targeted agents to inhibit more than one pathway.

Among agents that target VEGFR in the treatment of advanced NSCLC, axitinib [138] has resulted to be a potent selective inhibitor of these types of receptors; also cediranib [139] has been assayed in combination with carboplatin and PTX in the treatment of this kind of cancer.

Over-expression of EGFR has been associated with angiogenesis and poor prognosis in NSCLC [160]. Cetuximab (Erbiux®, ImClone/Bristol-Myer Squibb, USA) is a chimeric mAb that targets the EGFR pathway by binding to the extracellular domain of the receptor and in this way inhibiting the receptor-associated tyrosine kinase (TK) activity [140]. Furthermore, inhibitors of TK activity of EGFR have been used for targeting the receptor pathway. Small-molecule, such as erlotinib (Tarceva®, Genentech/Roche, Switzerland) and gefitinib (Iressa®, AstraZeneca plc, UK), compete reversibly with ATP to bind to the intracellular catalytic domain of EGFR TK and, thus, inhibit EGFR autophosphorylation and downstream signaling [161].

IGF-1R is a key signaling pathway that leads to the growth and survival of tumor cells [162] and is commonly overexpressed in lung cancer cells. Figitumumab (CP-751,871, Pfizer, USA) is a fully human monoclonal antibody that is a specific and potent inhibitor of IGF-1R. In combination with carboplatin/PTX, figitumumab has shown to be a promising antitumor agent as first line treatment of NSCLC [142]. Several other anti-IGF-1R mAbs are being investigated in the treatment of advanced NSCLC, among them IMC-A12 (cixutumumab; fully human IgG1 monoclonal antibody), MK0646 (dalotizumab; a humanized IgG1 monoclonal antibody) and R1507 (fully humanized monoclonal antibody) [163].

Lung cancer is a heterogeneous disease with multiple mutations, and it is unlikely that any single signaling pathway drives the oncogenic behaviour of all tumors. In fact, multilevel cross-stimulation among the targets of the new biological agents can contribute to the relative failure of some target therapies. In this way, combining targeted therapies is a promising research approach to the treatment of NSCL, and an exhaustive review has been recently published by Custodio and coworkers [163]

4.2. Colorectal cancer

The systemic treatment of metastatic colorectal cancer (mCRC) involves the use of active cytotoxic drugs and biological agents either in combination or as single agents.

Initial chemotherapy of mCRC is based on using several cytotoxic regimens [164]. These clinical trials are based on the results of key phase III studies conducted over the past

decade. The IFL regimen [irinotecan (I), 5-FU and leucovorin (LV)] has been extensively used [143]. Furthermore, combination of oxaliplatin and 5-FU/LV (FOLFOX4) [165] has improved the overall survival of mCRC patients.

A significant percentage of patients with CRC receive a biological agent targeting the vascular endothelial growth factor A (VEGF-A) or EGFR over their treatment course. The currently available anti-VEGF-A agent is bevacizumab (Avastin®, Roche-Pharma AG, Germany), a humanized mAb. Different key clinical trials incorporating bevacizumab have been carried out. In the AVF2107 trial [144] the combination of IFL and bevacizumab improved the progression-free survival and the overall survival. The trial of bevacizumab plus oxaliplatin-based chemotherapy (FOLFOX4) or plus capecitabine/oxaliplation (XELOX) [143,145] showed a significantly increase in the progression-free survival, mainly with XELOX.

The anti-EGFR mAbs indicated for mCRC treatment are cetuximab (Erbiux®, ImClone/Bristol-Myer Squibb, USA; a chimeric monoclonal antibody) and panitumumab (Amgen Inc; Thousand Oaks, USA; a fully human monoclonal antibody). Both of them are efficacy in the treatment of patients whose mCRC tumors express wild-type KRAS. Different clinical trials combining anti-EGFR agents and chemotherapy have been carried out. Thus, mCRC therapy (wild-type KRAS patients) based on cetuximab and FOLFIRY (CRYSTAL trial) [146] showed a significantly improved progression-free survival and overall survival. In a similar way, the combination of panitumumab and FOLFOX4 (PRIME trial) had a very positive impact on survival parameters in wild-type KRAS patients [147].

4.3. Breast cancer

Breast cancer is the most common cancer affecting females and one of the main causes of mortality of women. This disease shows a high heterogeneous nature in terms of genetic features, molecular profiles and clinical behaviour. The high mortality caused by breast cancer can be attributed to the development of metastatic breast cancer [166]. The discovery of "genetic signatures" in breast cancers can provide key insights into the mechanisms underlying tumorigenesis and can be proven useful for the design of targeted therapeutic approaches [167-168].

The HER2 is over-expressed 15-30% of invasive breast carcinomas [167]. Extracellular domain of HER2 has been the target of several monoclonal antibodies created in order to inhibit the proliferation of human cancer cells. Transtuzumab, a recombinant humanized anti-HER2 monoclonal antibody was approved by the FDA for immunotherapy of women with metastatic HER2 over-expressing breast carcinoma. This antibody provokes cell cycle arrest during G1 phase [148]. Transtuzumab has been extensively used to target different drug-loaded nanocarriers to breast cancer cells [169-170].

Many hormone receptor positive breast cancers are resistant to hormone therapies. Thus, clinical trials have been developed combining therapies with biological and targeted agents (anti EGFR and HER2) for the treatment of estrogen receptor (ER) positive breast cancer.

Combination of geftinib, an EGFR TK inhibitor, with anastrozole [171] or TMX [172] has conducted to a light prolongation of progression-free survival of patients. Clinical trials based on combination of transtuzumab and letrozole (Femara), an oral non-steroidal aromatase inhibitor for the treatment of hormonally-responsive breast cancer, in patients with ER+/HER2+ metastatic breast cancer have demonstrated clinical benefit [173]. A large proportion of HER2+ cancers have developed resistance to HER2-targeted therapeutics, including resistance of tumor cells to trastuzumab. Several agents have been developed to overcome resistance to this monoclonal antibody. The conjugation of maytansinoid DM1 with transtuzumab has generated transtuzumab-DM1 (T-DM1; Genetech Inc/Roche), that is active on HER2 overexpressing breast cancer and also transtuzumab-refractory tumors [149]. Another innovative targeted agent, which belongs, to the class of HER2-dimerization inhibitors, is pertuzumab (Omnitarg; Genentech/Roche), a recombinant humanized monoclonal antibody. Pertuzumab is directed against the highly conserved dimerization domain of HER2 [150]. The efficacy of adding pertuzumab to trastuzumab plus Dtxl for the first-line treatment of HER2-positive metastatic breast cancer was demonstrated in the randomized, double-blind, multinational, phase III CLEOPATRA trial [174].

Basal like breast cancers are the result of specific mutations. DNA lesions such as single-stand breaks (SSBs) and double-strand breaks (DSBs) are common in the normal cellular metabolism, and can be repaired by specific DNA repair mechanisms. In one of these DNA repair mechanisms, poly-(adenosine diphosphate ribose) polymerase 1 (PARP1) is an important key of the pathway. PARP1-inhibitors (PARP1-I) have been developed for the treatment of advanced breast cancer. Olaparib (AZD2281; AstraZeneca)), a PARP1-I has been evaluated in BRCA (a tumor suppressor protein) mutated patients [151]. Also iniparib (BSI 201; 4-iodo-3-nitrobenzamide; Sanofi-Aventis), an irreversible PARP1-I, is under study in patients with metastatic triple-negative breast cancer [175]. However, studies carried our by Liu and coworkers [176] shown that Iniparib nonselectively modifies cysteine-containing proteins in tumor cells, and the primary mechanism of action for iniparib is likely not via inhibition of PARP activity.

4.4. Prostate cancer

The current standard treatment of localized prostate cancer consists of prostatectomy and radiation therapy, sometimes supplemented with hormonal therapies to prevent testosterone production, which include anti-androgens and luteinizing hormone-releasing hormone (LH-RH) agonists. In locally advanced or widespread prostate cancer, the disease gradually transforms to a metastatic hormone-refractory state. Despite castrate levels of testosterone, the tumor will finally become independent of androgens resulting in death within a few years from diagnosis. In fact, the mortality rate of metastatic prostate cancer is extremely high. Thus, novel therapies [177-178] are on demand for the treatment of the malignant forms of prostate cancer that recur after initial therapies, including hormone refractory (HRPC) and castration resistant prostate cancer (CRCP).

Different molecules have been assayed as androgen and androgen receptor inhibitors. In this way, abiraterone acetate (Zytiga, Cougar Biotechnology) in combination with

prednisone has been recently approved by FDA for the treatment of CRCP in men who have received prior Dtxl chemotherapy [152]. Arbiraterone inhibits 17-α-hydroxylase, an enzyme of the testosterone biosynthesis pathway, decreasing circulating levels of the hormone. This enzyme is expressed in testicular, adrenal and prostate tumors. Regarding of androgen receptor inhibitors (AR-I), MDV3100 is an oral androgen receptor antagonist [153], which irreversibly binds to this intracellular receptor and causes no transcription of the gen.

The EGFR family (EGFR/HER) receptors have long been implicated in prostate cancer initiation and progression. EGFR is overexpressed in 18-37% prostate cancers [179], and a direct correlation of HER2 overexpression with the risk of death and recurrence in prostate cancer has been reported [180]. Thus, monoclonal antibodies have been studied as treatment options for prostate cancer. The efficacy of combining cetuximab with mitoxantrone plus prednisolone have been analyzed in a phase II clinical trial in men with CRCP after receiving Dtxl, but the time to progression and overall survival did not improve with the addition of cetuximab [154]. In order to evaluate whether dual inhibition of EGFR and HER2 would prolong the effectiveness of androgen withdrawal therapy (AWT) treatment in prostate cancer, studies using EGFR inhibitors (erlotinib and AG1478) and HER2 inhibitors (trastuzumab and AG879) were realized [155]. Results indicate that dual EGFR/HER2 inhibition, administered together with AWT, sensitize prostate cancer cells to apoptosis during AWT. In general, studies using inhibitors of EGFR/HER1 and HER2 show that these molecules fared poorly in prostate cancer clinical trials.

Recent research suggests that another family member HER3 (ErbB3) abets emergence of the castration resistant phenotype. The prostate cancer, in comparison to the normal tissue, overexpresses HER3 protein, which indicate poor prognosis. Antibody-based therapy that prevents ligand binding to ErbB3 appears promising and fully-humanized antibodies that inhibit ligand-induced phosphorylation of HER3 (ErbB3) are currently in early development [181]. HER3's signaling functions depend upon ligand binding to its extracellular domain and inhibitors are generated to disrupt this interaction. A recently-characterized, HER3-specific humanized antibody MM-121 blocked ligand-dependent HER3 activation induced by the HER1, HER2 or MET receptors [156].

The IGF-R signaling pathway plays a role in prostate cancer. In fact, an increase risk of prostate cancer has been directly correlated with the circulating IGF-1 (one ligand of the IGF-R) levels [182]. An inhibitor de the IGF pathway is the anti-IGF-R mAb cixutumumab (IMC-A12; ImClone Systems), which was effective in both androgen-dependent and androgen-independent human prostate cancer in animal models [183].

PSMA has been identified as an ideal antigenic target in prostate cancer. PSMA is the most well-established, highly restricted prostate cancer cell surface antigen. It is expressed at high density on the cell membrane of all prostate cancers, and after antibody binding, the PSMA-antibody complex is rapidly internalized along with any payload carried by the antibody. J591 is the first IgG mAb developed to target the extracellular domain of PSMA, and it has been deimmunized (humanized) to allow repeated dosing in patients. Three phase I studies have been carried out, two using the β-emitting radiometals yttrium 90 and lutetium 177

(177Lu), and a third using a cytotoxin (DM1) linked to J591 [157]. A phase II clinical trial (NCT00859781) to test the effectiveness of the radiolabel monoclonal antibody, 177Lu-J591 in combination with ketoconazole and hydrocortisone against prostate cancer is in progress.

5. Conclusion

The development of drug delivery systems that are able to modify the biodistribution, tissue uptake and pharmacokinetics of therapeutic agents is considered of great importance in biomedical research. Controlled release in drug delivery can significantly enhance the therapeutic effect of a drug. Among drug delivery systems, nanocarriers are the smallest devices for transport of drugs, and they comprises a variety of the type of nanoparticles developed for cancer, including liposomes, nanoshells, nanocapsules, dendrimers, polymer-drug conjugates, polymeric nanogels and micelles, and polynucleotide nanoparticles. The attractive properties of nanomedicines include their ability of controlled release of drugs, the targeting of specific tissues and the biocompatibility. Because of their size, nanocarriers can be taken up, in many cases, very efficiently by cells, internalized and stored into cytoplasm or different organelles. Nanocarrier uptake into a cell depends on the cell-type, since some cells are more susceptible to include nonfunctionalized systems via their design. The unique attributes of tumors support extravasation of polymeric nanomedicines through large pores on the endotheliallayer and via the disordered neoplastic tissue architecture. Thus, nanoparticles target the tumor passively via the EPR effect if their size is smaller than 100nm. Therefore, current research involves novel strategies to attach targeting ligands with high affinity for receptors overexpressed on tumors or ways to utilize the tumor´s own microenvironment as a stimulus for drug release. An active targeting strategy can improve the efficacy of the therapy and diminish side effects associated with drugs, since not all nanocarriers can overcome the cell membrane barrier without a targeting motif. Nanoparticle systems are able to target various portions of the tumor using specific targeting moieties and evade the problems associated with multi-drug resistance. Thus, to increase the delivery of a given drug to a specific target site, targeting ligands are conjugated to carriers. The presence of reactive pendant groups in nanogels make easy their vectorization forward specific cell motif by binding of ligands. Furthermore, it is an important fact that targeting ligands lead to macrophage recognition and faster clearance compared to the non-targeted nanoparticles. Various molecules, that include folates, transferrin, antibody and antibody fragments, peptides, aptamers, small molecules, and carbohydrates, have been used to target nanocarriers to specific receptors on tumoral cell surfaces. In many cases, ligand-targeted nanoparticles demonstrate better internalization by cancer cells and more effective intracellular drug delivery than other preparatios. The search for more molecular targets will advance the ability to improve delivery at the tumor level while decreasing toxicity to normal tissue. As a result, moieties-targeted drug-loaded nanoparticles, searching for new tumor targets, novel ligands, new strategies for targeting, and particle stabilization, are generally considered as promising candidates for cancer chemotherapy and we can expect their extensive clinical evaluation in the near future.

Nomenclature

2AAECM: *tert*-butyl-2-acrylamidoethyl carbamate

5-ALA: 5-aminolevulinic acid

5-FU: 5-Fluorouracil

7α-APTADD: 7α-(4′-amino)phenylthio-1,4-androstadiene-3,17-dione)

ABIR: $\alpha_v\beta_3$ integrin receptor

AD: adamantane

Apt: aptamer

AR-I: androgen receptor inhibitor

AWT: androgen withdrawal therapy

BBB: blood brain barrier

BCRP: breast cancer resistance protein

CEM: leukemic lymphoid cells

CRCP: castration resistant prostate cancer

CS: Chitosan

Dox: doxorubicin

DSBs: double-strand breaks

Dtxl: Docetaxel

ECM: extracellular matrix

EGF: epidermal growth factor

EGFR (or HER1): epidermal growth factor receptor 1

EPR: Enhanced Permeability and Retention

ER: estrogen receptor

EU: European Union

FA: Folic acid or folate

FDA: Food and Drug Administration

FR: folate receptor

FSiNPs: Fluorescent silica nanoparticles

GPI: glycosyl-phophatidylinositol

HB-EGF: heparin-binding epidermal growth factor

HCPT: Hydroxycamptothecin

HER: Human epidermal receptor

HER2: epidermal growth factor receptor 2

HER3: epidermal growth factor receptor 3

HFF: Human foreskin fibroblasts

HNSCC: Head and neck squamous cell carcinoma

HPAE-co-PLA/DPPE: hyperbranched amphiphilic poly[(amine-ester)-co-(D,L-lactide)]/1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine copolymer

HRPC: hormone refractory prostate cancer

HSA: human serum albumin

I: irinotecan

IFL regimen: irinotecan + 5-FU + leucovorin

IFP: interstitial fluid pressure

IGF: insulin-like growth factor

IGF-1R: insulin-like growth factor 1 receptor
LH-RH: luteinizing hormone-releasing hormone
LV: leucovorin
mAb: monoclonal antibody
MAPK: mitogen-activated protein kinases
mCRC: metastatic colorectal cancer
MePEG-PHDCA: poly(methoxy-polyethylene glycol cyanoacrylate-co-hexadecyl cyanoacrylate)
MTX: Methotrexate
MUC1: mucin 1
NCs: nanoclusters
NIPA: *N*-isopropylacrylamide
NPAM: *N*-(pyridin-4-ylmethyl)acrylamide
NPs: nanoparticles
NRs: nanorods
NS: niosomes
NSCLC: Non-small cell lung cancer
PAMAM: Poly(amido amine)
PARP1: poly-(adenosine diphosphate ribose) polymerase 1
PARP1-I: poly-(adenosine diphosphate ribose) polymerase 1 -inhibitors
PDGFR: platelet-derived growth factor receptor
PEG: polyethylene glycol
P-gp: P-glycoprotein
PHBHHX: poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)
PLGA: poly(D,L-lactide-coglycolyde)
PMPI: p-maleimidophenyl isocyanate
PRINT technology: Particle Replication In Non-wetting Templates technology
PSMA: Prostate-specific membrane antigen
PTK7: protein tyrosine kinase-7
PTX: Paclitaxel
RES: reticuloendothelial system
RGD: tripeptide arginine–glycine–aspartic acid
SELEX: Systematic Evolution of Ligands by Exponential Enrichment
siRNA: short inhibitory RNA
SSBs: single-stand breaks
SWNTs: single-wall carbon nanotubes
Tf: Transferrin
TfR: Transferrin receptor
TGF-α: transforming growth factor-α
TK: tyrosine kinase
TLR9: Toll-like receptor 9
TMX: Tamoxifen
TNF: tumor necrosis factor
VEGF: vascular endothelial grown factor
VEGFR: vascular endothelial grown factor receptor

VPF: vascular permeability factor
WHO: World Health Organization

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