Actively targeted nanocarriers for drug delivery to cancer cells

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Abstract

Introduction: The progressive breakthroughs in nanomedicine have been instrumental for the clinical translation of actively targeted drug-delivery approaches. Besides storing large payloads of drugs within the nanoparticle core, the conjugation of targeting moieties confers specific targeting ability to the drug delivery nanoplatforms. In this respect, clinical results suggest that actively targeted nanocarriers can exhibit an overall improved antitumor efficacy, minimizing off-target toxicity.

Areas covered: This review article summarizes the advances in active targeting of nanocarriers to cancer cells. Specifically, we discuss the various types of nanocarriers, describe the receptors that are frequently overexpressed in solid tumors, and discuss how this approach can be used to improve clinical outcomes. We particularly focus on ongoing clinical trials of actively targeted nanoparticles that are yet to be clinically approved.

Expert opinion: The motivation to further invest in active targeting will likely pose to the clinical benefits observed with the first generation of targeted nanoparticles. We envisage a future requiring the use of longitudinal measures in the clinical setting to profile the patients that are likely to benefit from actively targeted nanocarriers. At the preclinical stage, a complete picture of intratumoral barriers combined with a quantitative approach of the intratumoral fate of nanomaterials will be instrumental in defining more effective strategies to improve their clinical translation.

Keywords: nanoparticles, targeting, drug delivery, cancer cells, receptors

Article highlights:

- Active targeting of nanocarriers offers the opportunity to deliver a specific drug to a specific cell resulting in enhanced efficacy and reduced side effects.
- Even if the nanocarrier is designed for active targeting to arise, at first passive accumulation (EPR effect) occurs followed by target-specific binding as a complementary strategy.
- The extent of EPR effect is influenced by the heterogeneity of tumors and dynamic status of each tumor that need to be further investigated.
- Nanoparticles engineering has evolved rapidly in the last decade and their contribution to cancer research is increasing in parallel, facilitating the preclinical to clinical translation of selected actively targeted nanocarriers.
- Recent evidences from preclinical studies indicate that exosomes represent one of the most promising innovative drug delivery system for improving traditional chemotherapy, gene silencing as well as immunotherapy of tumors.
- EGFR, PSMA, transferrin and folate receptors are the most advanced active-targeting strategies based on the concept that these antigens are overexpressed on cancer cell membranes.
- At present, few nanoparticle types are in clinical trials for cancer drug delivery, mainly formulated with liposomes or polymeric materials, however new kinds of nanoparticles are expected to enter clinical trials in the near feature.
- Exciting results are emerging from Phase I and II clinical trials of targeted retroviral nanoparticles.

1. Introduction

Cancer is a leading cause of death worldwide, accounting for an estimated 9.6 million deaths in 2018, and it has a major impact on society in terms of loss of productivity and poor health-related quality of life [1]. Current treatment for cancer relies on various combinations of surgery, chemotherapy, radiotherapy and hormone therapy, and many patients under ongoing therapies suffer a poor quality of life during and after the treatment [2]. Using the Global Burden of Disease (GBD) methodology, it has been estimated that in 2015, there were 17.5 million cancer cases, 8.7 million deaths, and 208.3 million disability-adjusted life-years (DALYs) as a measure of overall disease burden [3].

Despite the intensive efforts to impact on the cancer death rate, the goals of the effectiveness of metastatic disease treatment and tolerability regarding short- and long-term side effects remain elusive [4, 5]. Therefore, it is strictly necessary to reduce the side effects of chemotherapeutic agents to healthy tissues, primarily in the bone marrow and gastrointestinal tract, and to have a better targeting/localization of therapeutics. According to Ehrlich's 'magic bullet concept', targeted drugs that go straight to their intended cell-structural targets, should in principle efficaciously affect cancer cells yet remain harmless in healthy tissues [6].

Of interest, nanomedicine for cancer therapy is advantageous because it has the potential to deliver the nanoparticle to tumors owing to the enhanced vascular permeability and retention (EPR) effect which is referred to as passive targeting [7]. However, passive targeting suffers from the severe limitations such as significant heterogeneity within and between tumor types (including primary tumor versus metastatic lesions), inefficient drug uptake and retention into tumor cells, and the lack of EPR effect in some tumors [8]. Further translational studies and clinical trials would enable a better understanding of the mechanisms that underlie EPR function in a tumor and its primary or metastatic sites and may allow effective nanodrugs to be optimized.

Among the most promising way to solve the problems of passive targeting, the development of actively targeted drug-delivery approaches has been supported by the progressive breakthroughs in

nanomedicine [9]. Besides developing nanocarriers systems, which allow storing large payloads of drugs within the nanoparticle core, the conjugation of targeting moieties can confer specific targeting ability to the drug delivery nanoplatforms [10]. Even if the nanocarrier is designed for active targeting to arise, at first passive accumulation occurs followed by target-specific binding as a complementary strategy. The possibility of improved targeting results in greater efficacy of the treatment and less side effects caused by the accumulation of the drug in healthy tissues. Therefore, the concept of actively target nanotherapeutics has been gaining great interest as a promising approach to address unmet medical needs [11,12].

The present study is intended to provide a general overview of the different targeting strategies. The opportunities and challenges achieved by using different targeting molecules will be highlighted, as well as the current clinical trials for nanoparticle formulations that have yet to be clinically approved.

2. Nanoparticles used for active targeting

For an efficient anticancer therapy, the rationale for the design of a bioactive nanoparticle system should take into consideration several aspects, starting from the chemical-physical parameters of the nanoparticles, the biology of the tumor to be targeted, the characteristics of the active molecule to be linked. Nanoparticles engineering has evolved during decades reaching the up-to-date state of the art with the mindfulness that several major chemical-physical parameters, that we briefly review here, have an impact on the efficiency of the vehicle and overall on the therapeutic efficacy against cancer.

2.1 Role of the size

The size has an important role in the biodistribution, in the clearance fate, in the cell entry and in the intracellular pathway activation. Generally, it is well known that larger nanoparticles can entry more efficiently into cells thanks to clathrin-mediated uptake and that smaller nanoparticles tend to clamp together depending by the pH of the surrounding environment, behaving like bigger

nanoparticles when they can aggregate. Less obvious is the fact that smaller nanoparticles (with diameters smaller than 6-8 nm that is the threshold for renal filtration) have a faster renal clearance and then can be quickly removed from the circulation, with a huge limitation of their active cancer targeting [13]. Despite this, a recent research demonstrated that ultrasmall (6-7 nm in diameter) targeted nanoparticles can be functionalized to exhibit high tumor-targeting efficiency and efficient renal clearance, bypassing the issues about the use of small nanoparticles for nanomedicine [14]. These data demonstrated a high capacity of penetration, specific accumulation and retention of the ultrasmall nanoparticles into the tumoral tissue *in vivo* [14], opening new opportunity to engineer precisely controlled and scalable ultrasmall particles with broad utility, including specific drug delivery and/or specific imaging (depending on the incorporated payloads).

2.2 Importance of the surface charge

The surface charge has fundamental impacts on solubility, stability, biodistribution and cellular uptake/entry. In particular, several authors have shown that positive-charged nanoparticles have a higher capacity of surface adsorption, uptake and entry due to the negative charge of the cell membrane, while the same nanoparticles characterized for having negative surface charge perform significantly less. This was shown for several types of platforms including silver [15] and silica nanoparticles [16]. Importantly, functionalization with targeting proteins seems to have little effects on changing the intensity of charge attraction between nanoparticles and cell surface, while having obviously a role in the specific identification of the surface target on the cell membrane [17]. A recommendation would be to use always a backbone of cationic nanoparticles to enhance cell adhesion and entry, independently by any further specific functionalization. The presence of an active targeting moiety is by the way able to address the intracellular effects as was recently showed by Clemons et al. The authors showed in cellular models that the therapeutic efficacy of nanoparticles decorated with GE11 peptide, for active targeting EGFR, were able to significantly enhance the therapeutic effect of the loaded drug (docetaxel), compared to the non-targeted formulation [18]. The authors demonstrated that this difference was due predominantly by a

modified internal trafficking following the activation of the EGFR intracellular pathway that allows a different processing of the cargo drug and a better therapeutic response. In particular, they observed that the EGFR targeted nanoparticles associated significantly less with endosomes and lysosomes. This result indicates that beyond the cellular uptake, the active targeting of nanoparticles changes also the intracellular fate of the cargo drug, impacting positively in the therapeutic efficacy. A message from this interesting research is that a fine use of the active targeting could overcome the limitations of a backbone of anionic or neutral nanoparticles, widening the surface charge range usefull for creating efficient nanoparticles.

2.3 Role of the shape

The shape of the nanoparticles affects both intrinsic features, such as the surface area available for binding of the specific targeting molecule (or drug), as well extrinsic features, such as the circulation, biodistribution and accumulation in the body [19,20]. Sometimes the tridimensional structure of the nanoparticles is obligated by their chemical composition, such as for liposomes or micelles that can assume only spherical conformation. When the row material, such as gold, allows to create nanoparticles with different shape, several researchers have demonstrated that spherical nanoparticles possess the best profile in blood/organic fluid flowing as well as in the interaction with target cells and finally in the cell entry, respect for example to rod, star and hollow shapes [21]. Nevertheless, this is still an open debate since other authors have found that spherical nanoparticles are not always the best performers in specific contexts [22,23]. These studies probably suffer from some limitations such as the different size of the compared nanoparticles, however further investigations are needed to better address this issue.

2.4 Traditional and innovative nanoparticles for cancer active targeting research

In addition to chemical-physical parameters, evaluation of toxicity and biodegradability/biocompatibility are of course features that concur heavily in discriminating about the approval for human destination of the designed nanomedicine (by the FDA or equivalent European and/or Chinese organizations). The intrinsic nature of the nanoparticles (row materials

composition) can have an important impact in overall biocompatibility and safety of the nanoparticles. At present, a low grade of toxicity is reached by PLGA polymeric nanoparticles [24], but innovative nanoparticles are now entering clinical trials, as discussed later, and will offer new perspectives on cancer treatment. Another aspect to be considered for translation to the clinic is the easiness of production that includes large scale reproducibility and economic feasibility. These points are of course limiting the last phase of diffusion and commercialization.

Considering the above parameters affecting the overall efficacy of nanoparticles, at present, liposomes, polymeric nanoparticles, micelles, mesoporous silica nanoparticles and gold nanoparticles are the preferred used nanoparticles in cancer research and the most advanced to the clinic; other systems include iron oxide nanoparticles, dendrimers, carbon nanotubes and, more recently, nanoheterostructures (NHS) and artificial biomimetic exosomes (**Table 1**).

In particular the latter, composed by plasma membrane-like phospholipids and membrane-anchored proteins and for this reason called "biomimetic", are gaining increasing interest in the field of cancer active targeting and immunotherapy and have been recently recognized by several authors as the most promising drug delivery system, even if some drawbacks still need to be addressed before their clinic translation [43]. It has to be mentioned that artificial biomimetic exosomes have a different origin respect to circulating "physiological tumor-made" exosomes that are being investigating as prognostic or diagnostic markers following liquid biopsy. Artificial biomimetic exosomes are currently prepared from cell cultures (top-down methods) or from chemical artificial bilayers (bottom-up methods) [43]. During the preparation process they can be engineered to exhibit receptors to target specific antigens and/or to encapsulate chemotherapeutics or siRNAs to enhance drug delivery to cancer cells thanks also to the presence of trans-membrane and membrane-anchored proteins that may enhance endocytosis. Recently, Kamerkar S et al published interesting evidences about the capacity of engineered exosomes (called iExosomes) to target oncogenic KRAS in pancreatic cancer models *in vitro* as well as *in vivo* in orthotopic tumors and in genetically modified mice (KTC and KPC mice) [40]. The iExosomes were characterized on the

surface by CD47 molecules able to avoid clearance by monocytes thanks to a "don't eat me signal" and to permit retention of exosomes in the circulation of mice favoring tumoral cells entry. Moreover, they were also engineered to carry short interfering RNA for specific targeting the oncogenic $KRAS^{G12D}$. This strategy resulted in the suppression of pancreatic cancer and significant increase in the overall survival of mice treated with iExosomes, showing that iExosomes are a real opportunity for active tumor targeting. This research was shortly followed by the publication of another paper demonstrating the clinical-grade production of the same iExosomes and highlighting that the clinical translation of exosomes is possible and close [44].

3. Active targeting strategies

Conventionally, actively targeted nanocarriers have an advantage over their non-functionalized counterparts by being more effective at the site of drug delivery and also reducing any potential off-target toxicity. Even if the nanocarrier is designed for active targeting to arise, a first passive accumulation occurs followed by target-specific binding as a complementary strategy [45]. However, the relative contribution of active and passive tumor targeting is not fully characterized, and further studies on the mechanisms underlying these dynamics are highly required [45,46]. Although there seems to be a clear EPR effect in clinical tumors, which potentially improves the NP ability to interact with target cell receptors, the extent of EPR effect is influenced by the heterogeneity of tumors and dynamic status of each tumor. Therefore, there is a definite need for systematic investigation of factors that could affect the transport of nanocarriers to a target site after intravenous administration as well as events underpinning the response toward a specific drug delivery system [47, 48, 49].

Overall, a variety of technological issues and factors such as formulations, efficiency of drug loading into delivery vehicle, residence in the circulation and drug release at the site need to be addressed to develop improved targeted drug delivery systems. In nanoparticle design, different parameters must be taken into account when choosing an appropriate active molecule to be linked,

the most relevant being up-regulation of the complementary receptor/antigen in cancer cells compared to normal tissues. The presence or absence of the targeted receptor is directly related to the response rates of active targeting therapies [50]. Consequently, reliable methods for detecting receptor-positive tumors are needed for the careful selection of patients who are likely to benefit from targeted drug delivery. In light of this, most of the receptors currently under investigation in nanomedicine are those towards which effective and selective therapeutic agents have already been developed as free drugs (i.e. targeted therapeutics) [50]. As newly emerging treatment lines, targeted therapeutics have indeed provided significant improvements in several clinical trials, paving the way for personalized medicine and serving as a model for active targeting strategies. The following section comprises a representative selection of specific targets from a vast field of receptor/antigens that have been investigated to design active-targeting nanocarriers.

3.1 Human epidermal growth factor receptor family

The Human Epidermal Growth Factor Receptor (HER) family includes four members, epidermal growth factor receptor (EGFR or HER1), HER2, HER3 and HER4 [51]. These structurally related receptors are composed of an extracellular ligand-binding domain, a hydrophobic transmembrane region, and an intracytoplasmic tyrosine kinase domain [52]. EGFR and its signaling pathway have been studied extensively owing to their role in normal physiology and in the pathogenesis and progression of various cancers [53]. Based on the observation that the EGFR was frequently overexpressed in epithelial tumors and on the preclinical evidence of anti-EGFR mAb activity, EGFR was the first receptor to be proposed as an attractive therapeutic target for cancer treatment. After 2 decades of intensive research, there are several EGFR-targeted therapeutics available in the clinic [52]. In particular, the design of affibody molecules, antibodies and small-molecule drugs targeting the EGFR receptor has represented promising alternative strategies to improving clinical outcome [54]. Although EGFR signaling plays a critical role in tumor development and progression in a variety of cancers, agents targeting EGFR are variably effective depending on the underlying tumor type. This may depend on the molecular characteristics of different tissues, environmental

factors, and host genetic background. For instance HER2 has served as a prognostic and predictive biomarker in breast and gastric/gastroesophageal cancers, whereas it is still being investigated as a potential therapeutic target in ovarian cancer. Recent advances in our understanding of the mechanisms that underlie this receptor overexpression and function, identification of EGFR somatic mutations, as well as new clinical trials on anti-EGFR agents provide additional information on the clinical targeting of this receptor [52].

Currently, four EGFR-targeted nanocarriers for cancer therapy are in clinical trials (details will be summarized in the later part of this review) and significant efforts continue to explore them, as well as developing new ones at the preclinical stage. One such example developed by Du C *et al.* is the EGFR targeting self-assembly amphiphilic peptide nanoparticle loaded with gemcitabine and poly-ADP-ribose polymerase inhibitor (PARPi), designed to improve the chemotherapy in pancreatic cancer patients with BRCA mutations which may benefit from PARPi inhibitors. The nanoparticle prolonged the half-life of both drugs and resulted in their tumor co-delivery at an adequate concentration in a murine pancreatic cancer model. This markedly inhibited tumor growth with minimal side effects and represents a promising approach for treatment of pancreatic cancers with BRCA mutations [55]. In another study cetuximab-guided Avidin-Nucleic-Acid-Nano-Assemblies (ANANAS) carrying doxorubicin internalized EGFR-expressing cells more efficiently than the antibody alone [56]. The *in vivo* results showed that NP-linked cetuximab outperformed the corresponding antibody-drug conjugate in a model of human EGFR positive triple-negative breast cancer leading to 43% tumor reduction at low drug dosage (0.56 mg/kg). This notable potency in a tumor resistant to cetuximab or doxorubicin treatments as such was ascribed to the EGFR targeting/binding property, which was capable to bypass drug resistance. This results figure out the importance of surface composition in the active-targeting nanoparticle design and could be further expand application of the anti-EGFR antibody to a wider number of cancer patients currently suffering from poor prognosis. Among the examples of the most promising nanoplatforms, a multivalent bi-specific nanobioconjugate engager (mBiNE), specifically designed for immune-

mediated eradication of HER2-expressing tumors, has been developed by simultaneously targeting the HER2 expressed by cancer cells and pro-phagocytosis signalling mediated by calreticulin [57]. Notwithstanding the initial receptor-mediated immune activation by the mBiNE, the subsequent antitumor immunity also induced protective effects against HER2-negative tumor cells. As a consequence, the mBiNE represents a new tool to stimulate innate and adaptive immunity and promote an innovative broadly active immunotherapy.

Chapter highlights

- EGFR is frequently overexpressed in epithelial tumors.
- EGFR was the first receptor to be proposed as an attractive therapeutic target for cancer treatment.
- Identification of EGFR somatic mutations, as well as new clinical trials on anti-EGFR agents provide additional information on the clinical targeting of this receptor.
- Four EGFR-targeted nanocarriers for cancer therapy are currently in clinical trials.
- New promising nanoplatforms simultaneously target the HER2 expressed by cancer cells and stimulate innate and adaptive immunity.

3.2 Prostate-specific membrane antigen

Prostate-specific membrane antigen (PSMA) is a type II transmembrane glycoprotein with folate hydrolase activity that is highly expressed in prostate cancer cells compared to normal non-prostatic tissue. PSMA tissue distribution in the normal human prostate is relatively restricted to the cytoplasm and apical side of the epithelium surrounding the prostatic ducts [58]. PSMA also is up-regulated in the tumor-associated endothelial cells of many different solid tumors, and therefore has been extensively investigated as a potential antigen for direct tumor-specific vascular targeting [59,60].

In light of the observation that neo-vasculature play an important role in the development and progression of solid cancers and that inhibition of PSMA's enzymatic activity could severely impair the formation of new vessels, a series of recent studies have demonstrated the utility of engineering

drug loaded nanoparticles with PSMA-ligand. Li *et al.* developed a doxorubicin–polylactide nanoconjugate functionalized with an aptamer against PSMA and demonstrated its ability to enhanced cell death and necrosis in a canine hemangiosarcoma model [61]. This is an ideal comparative solid tumor model system as it is composed solely of tumor-associated endothelium. As expected, the nanoconjugate selectively targeted PSMA-expressing tumor-associated endothelial cells, exhibited a substantial cytoreductive activity and a desirable safety profile. Another promising class of PSMA-targeted nanoparticles that is generating interest to effectively treat various solid malignancies is that of docetaxel-loaded polymeric nanosuspensions based on polylactic acid-polyethylene glycol block copolymer (PLA-PEG) [62]. These nanosuspensions possess a superior efficiency and fewer off-target side effects in the treatment of gastroesophageal and breast cancers.

In the context of prostate cancer therapy, several active-targeted nanocarriers have been developed as theranostic platforms for assisting in precision surgery and enable an optimal prostatectomy. Early studies on a series of antibody-conjugated iron oxide nanoparticles which target PSMA were carried out *in vitro* [63]. This technology combines the binding selectivity of antibodies and the theranostic properties of iron oxide nanoparticles, which enable MRI imaging (owing to their magnetic properties) and therapy (through hyperthermia or drug delivery). Mangadlao *et al*. have developed a theranostic agent based on PSMA-targeted gold nanoparticles loaded with a fluorescent photodynamic therapy (PDT) drug, Pc4 [33]. *In vitro* tests showed that these nanoparticles enabled to achieve simultaneously an excellent specific uptake in PSMA-positive cells, a suitable imaging contrast and a more complete cell killing upon PDT. Of note, *in vivo* studies showed remission on PSMA-expressing tumors 14 days upon exposure to light.

Though the examples of PSMA targeting ligands such as anti-PSMA antibodies and PSMA aptamers are more extensive, there are nevertheless PSMA-targeted nanocarriers that utilize folic acid as a targeting ligand. For instance, Flores and colleagues showed that a multivalent folate-conjugated nanocarrier that encapsulated a cytotoxic, cancer-specific peptide (CT20p) can induce

cell death to PSMA expressing prostate cancer tumors, owing to the PSMA ability to bind and internalize folate-conjugate compounds [64].

Polymeric nanoparticle targeting prostate-specific membrane antigen loaded with docetaxel (BIND-014) have entered clinical trials, as discussed in the later part of this review.

Chapter highlights

- Neo-vasculature play an important role in the development and progression of solid cancers and inhibition of PSMA's enzymatic activity can severely impair the formation of new vessels.
- Canine hemangiosarcoma model is an ideal comparative solid tumor model system as it is composed solely of tumor-associated endothelium.
- Theranostic nanoplatforms could assist in precision surgery and enable an optimal prostatectomy.
- PSMA-targeted nanocarriers can utilize folic acid as a targeting ligand.
- Polymeric nanoparticle targeting prostate-specific membrane antigen loaded with docetaxel (BIND-014) have entered clinical trials.

3.3 Transferrin receptor

The human transferrin receptor (TfR1) also known as CD71, is a type II transmembrane glycoprotein expressed on the cell surface, involved in the iron homeostasis and in the regulation of the cellular growth. It consists of two monomers linked by two disulfide bonds (190 kDa) each containing a large extracellular C-terminal domain, a single pass transmembrane domain and a short intracellular N-terminal domain [65,66]. TfR1 binds the plasmatic diferric transferrin for which it has a nanomolar affinity and the ligand-receptor complex is internalized through clathrin-mediated endocytosis [66]. In human there is a second transferrin receptor (TfR2) that is expressed mainly in the hepatocytes and erythroid precursor cells [67].

TfR1 owes its promise as a target for drug delivery in cancer cells to its high expression level, extracellular accessibility, the ability to be internalize and its role in neoplastic processing [65]. The

TfR1 is abundantly expressed in a large number of cancers including liver, breast, brain, lung, prostate, ovarian and colon cancer cells; its expression in these cancers can be up to 100-fold than in normal cells [65,66,68]. Also, in human leukemia there are high levels of TfR1 expression in T lineage cells and mature B lineage ALL cells [66]. Moreover, since TfR1 is expressed in the brain capillary endothelial cells of blood-brain barrier (BBB), it is a target for the development of nano-chemotherapy drugs to improve delivery to the brain as well [69]. As schematized in **Figure 1**, the intracellular iron concentration regulate the TfR1 gene expression, and consequently the correct balance between intake, utilization and efflux of iron maintains homeostasis in the cells. When intracellular labile iron pool reaches high concentrations due to excessive acquisition and retention, it triggers production of free radicals that damage biomolecules and increase oxidative stress, inducing tumorigenesis [66,70]. Moreover, in response to inflammation, hypoxia, oxidative stress and mutations of the regulator genes, an increased expression of TfR1 may occur, affecting tumor proliferation, migration, invasion, apoptosis and metastasis [65] (**Figure 1**).

TfR2 is also expressed in colon cancer, ovarian cancer, glioblastoma and lymphoma cell lines. Of interest, under conditions of hypoxia Glioblastoma multiforme (GBM) TB10 cell lines showed high expression of TfR2, which was probably linked to high rates of cell proliferation and suggested a role of TfR2 in the tumor's angiogenesis [71].

These abovementioned characteristics make TfRs interesting targets in the development of ligands-based nano-therapies using natural ligand transferrin (Tf), monoclonal antibodies (mAbs) or single chain variable fragments (scFv). As previously mentioned, since Tf conjugates interact with both TfR1 and TfR2 receptors, mAbs directed against TfR1 could confer selectivity to the construct while avoiding toxicity in the hepatocytes [72]. **Table 2** shows a panel of representative nanocarriers that employ ligands that interact with TfR1 for active cancer targeting.

Two liposomal systems (MBP-426 and SGT-53) designed to target TfR have entered clinical trials [72]. MBP-426 is an oxaliplatin-loaded liposome conjugated with Tf that reached a Phase Ib/II clinical trial in patients with second line metastatic gastric, gastro-oesophageal junction and

oesophageal adenocarcinoma (2009, NCT00964080) [72]. The SGT-53, reported in **Table 2**, is a cationic immunoliposome conjugated with anti-TfR single chain Fv on the surface and carrying the plasmid DNA encoding human wild type p53 gene. This completed a phase Ia of human clinical trial and is undergoing clinical evaluation in Phase II (NCT02340156). CALAA-01 is another notable example of Tf-targeted polymeric nanoparticle for cancer chemotherapy to reach clinical Phase I trials (see Section 4).

Chapter highlights

- TfR1 owes its promise as a target for drug delivery in cancer cells to its high expression level, extracellular accessibility, the ability to be internalize and its role in neoplastic processing.
- TfR1-targeted nanoparticles have emerged as a promising strategy in brain cancer treatment as TfR1 is expressed in the brain capillary endothelial cells of blood-brain barrier (BBB).
- TfR2 can be a tumour biomarker but is expressed mainly in the hepatocytes and erythroid precursor cells.
- Two liposomal systems and a polymeric nanoparticle targeting TfR have entered clinical trials.

3.4 Folate receptor

Folates are essential vitamins that are important for DNA replication and cell division. Under physiologic conditions, exogenous reduced folates are predominantly transported into the cell cytosol via the low-affinity (KD ~1–5 μm) and ubiquitously expressed reduced folate carriers [83]. Folates are also transported by high-affinity (KD ~100 pm) folate receptors (FRs) which preferentially mediates the uptake of oxidized forms of folate (e.g. folic acid) into the cell by endocytosis [83].

In humans, there are four isoforms of the FR (Frα, FRβ, FRχ, FRδ) [84]. Notwithstanding FRβ's expression on some cancers and on tumor-associated macrophages of many cancers, the Frα isoform has the most potential for active targeting strategies. Normal tissue distribution of

FRα is relatively restricted to a limited number of polarized epithelia (uterus, placenta, choroid plexus, lung, and kidney), which expression localized only at the apical surface of polarized cells, preventing exposure to the circulation [84] (**Figure 2**). In the malignant context, intercellular junctions are lost and FRα loses its polarized cellular location, thus becoming positioned on the entire tumor cell surface (**Figure 2**). Due to the important role that FRα plays in cancer development and progression, this receptor is frequently highly overexpressed in several epithelial tumors, including a variable percentage of ovarian, breast, lung, kidney, and colon carcinomas [85]. This consequently enables the use of folate-targeting ligands to guide the receptor-mediated endocytosis of folate-targeted nanocarrier containing therapeutic payloads. Upon endocytosis, nanocarrier can release its content into the cytosol of the tumor cells. Indeed, several studies have been conducting to develop variousconjugates that employ a folate ligand for active targeting [86] (**Table 3**).

Disappointingly, to date no folate-based NPs have entered the clinic for cancer therapy. Lack of clinical translation may depend on factors such as the heterogeneous FRα expression on tumors and whether the molecular characteristics of tumors change over time. Noteworthy in this regard are conflicting literature reports on the prognostic and predictive significance of the FRα, as differences in survival rates associated with FRα expression have been shown in the clinic according to histology, stage and time following diagnosis [94,95]. In particular, a study showed that serous ovarian cancer patients with higher α-FR expressions in cancerous tissue had lower chemotherapeutic response, significantly decreased disease free interval and poorer overall survival which might be related to α-FR-induced anti-apoptosis [95]. Conversely, a successive study showed a survival advantage in the first 2 years following diagnosis for patients with FRα positive high-grade serous ovarian carcinomas compared with patients with FRα negative histology [94]. However, FRα positive analysis in patients with clear cell carcinomas was associated with shorter

progression-free survival interval [94]. Therefore, further histologic analysis should be prospectively associated with clinical outcome.

Chapter highlights

- In humans, there are four isoforms of the FR (Frα, FRβ, FRχ, FRδ), and the FRα isoform has the most potential for active targeting strategies.
- In the malignant context, FRα loses its polarized cellular location, thus becoming accessible to actively targeted nanoparticles in blood circulation.
- FRα is frequently highly overexpressed in several epithelial tumors.
- Despite promising preclinical results, to date no folate-based NPs have entered clinical trials for cancer therapy.
- Further studies on prognostic and predictive significance of the FRα are needed.

4. Targeted nanoparticles for cancer in the clinical trials

Although actively targeted nanocarriers are to be yet approved by the Food and Drug Administration (FDA), there are a few currently under clinical development that show particular promise as drug delivery enhancer for a variety of solid tumors (**Table 4, Figure 3**). The majority of actively targeted nanoparticles currently in clinical trials are either liposomal or polymeric. However, since a wide range of materials have been extensively investigated for active targeting in preclinical studies, new kind of nanoparticles are expected to enter clinical trials in the near future.

CALAA-01 was the first targeted, polymer-based nanoparticle for siRNA delivery to reach clinical development in 2008 [96]. Results from a human Phase I clinical trial showed that CALAA-01 was well tolerated during the initial dose escalation and indicated that the behavior of CALAA-01 in humans was adherent to what observed previously in animal models. Remarkably, the delivery system used in CALAA-1 translated into clinically meaningful data providing for tumor-specific delivery of functional siRNA, and representing the first example of dose dependent accumulation of

targeted nanoparticles in human tumors [97]. Results demonstrated that a potent and specific gene silencing (RNAi) can be achieved in human by a systemically delivered siRNA properly formulated.

BIND-014 is another notable example of targeted and controlled release polymeric nanoparticle for cancer chemotherapy to reach clinical Phase I trials in January 2011 [60,98]. Since the molecular target, prostate-specific membrane antigen (PSMA), is particularly expressed on prostate tumor cells as well as in the tumor-associated neovasculature of nonprostatic solid tumors, BIND-014 have the potential to improve the tumor-specific delivery of docetaxel in the treatment of a broad range of solid tumors. A Phase I study in a panel of solid tumors showed that BIND-014 was well tolerated, with transient and manageable neutropenia as the dose-limiting toxicity. Of note, the nanoplatform used in BIND-014 provided a synergic combination of long circulation effect, site-specific targeting, and controlled release of docetaxel, thus enabling to achieve tumor growth inhibition for an extended period of time. A Phase II clinical trial evaluating patients with metastatic castration-resistant prostate cancer (mCRPC) suggested that treatment with BIND-014 is active and well tolerated. PSMA expression levels on PSMA-positive circulating tumor cells (CTCs) may also be related to antitumor activity of BIND-014 thus allowing for the selection of patients who are likely to benefit from this treatment.

Rexin-G®, a tumor-targeted retroviral expression vector encoding an anti-cyclin G1 construct, is the world's first tumor-targeted injectable gene therapy vector that has been tested in the clinic and was granted orphan drug status for multiple cancer indications in the US [99]. The targeted retroviral nanoparticles bind to exposed collagenous (XC) proteins via a high-affinity collagen-binding motif derived from von Willebrand coagulation factor. According to the presence of XC proteins, Rexin-G® accumulates in areas of tumor invasion, neoangiogenesis and stroma formation.

The results of Phase 1 and Phase 2 clinical trials for sarcoma and pancreatic cancer demonstrated the overall safety and the dose-dependent effectiveness in controlling tumor growth and improving overall survival. Remarkably, it is reported the unique case of a 14 year-old female with metastatic

malignant peripheral nerve sheath tumor of the parotid gland, who has no signs of active disease 9 years after starting the Rexin-G® treatment as monotherapy [100]. To date, long-term follow-up observational studies, required by the US FDA for investigational gene therapy products, haven't reported any delayed or late adverse events associated with Rexin-G® treatment.

As an alternative to the liposomal or polymer-based nanoparticles frequently used to deliver microRNA (miRNA), bacterially derived 400 nm minicells produced by de-repressing polar sites of cell division in bacteria are described [101]. TargomiRs are minicells loaded with miR-16-based mimic miRNAs and targeted with an anti-EGFR-specific antibody, which are aimed to compensate the lack of some specific tumor-suppressor miRNAs in mesothelioma and lung cancer patients by replacing the lost miRNAs. The first-in-man, open-label, dose-escalation Phase 1 trial demonstrated the acceptable safety profile and early signs of activity of TargomiRs in patients with malignant pleural mesothelioma, thus supporting additional studies of TargomiRs in combination with chemotherapy or immune checkpoint inhibitors [102].

Another promising class of EGFR-targeted nanoparticles that is generating interest to effectively treat solid malignancies is that of doxorubicin-loaded liposomes based on antibody that target HER2-overexpressing tumor cells [103]. The results from the first-in-human phase 1 study of MM-302 in patients with advanced HER2-positive breast cancer suggest that MM-302 monotherapy, in combination with trastuzumab or trastuzumab and cyclophosphamide is active and has an acceptable safety profile, owing to a reduced exposure of doxorubicin to healthy cells such as cardiomyocytes [103]. Based on the observation that the EGFR is overexpressed in 40-50% of patients with recurrent glioblastoma (GBM) and on the limited treatment options available, a novel nanocellular (minicell) compound was designed to target EGFR-positive tumors via minicell-surface attached bispecific proteins [104]. The first in human Phase I study of these EGFR-targeted minicells patients with EGFR-positive GBM demonstrated a high tolerance profile with no dose limiting toxicity, thus supporting further investigation on these attractive therapeutic nanocarriers for GMB treatments.

5. Conclusion

The future direction for cancer medicine is a precise and personalized therapy. Nanoparticles engineering and preclinical research on this field have evolved rapidly in this direction proposing a vast panorama of different actively targeted nanocarriers. Advantages of the use of such strategies rely on protection of the cargo from the extra-nanoparticle milieu (blood circulation, tumor microenvironment, intracellular chemical/biochemical exposition), fine localization of the therapeutic and side-effect reduction on healthy tissues, enhancement of the therapeutic efficacy thanks to the activation of target-specific intracellular pathways. At present, important goals have been achieved in preclinical and clinical studies of several types of actively targeted nanoparticles, with exiting results arriving from the first clinical phases of the targeted retroviral nanoparticles Rexin-G®. Nonetheless, some limitations need to be solved, such as overall tolerability and biodegradation, and effects on metastasis are still rare evidences.

6. Expert Opinion

While clinically-approved nanoparticle-based drug delivery systems have shown consistent results concerning drug toxicity reduction, their use has not always shown improvements in clinical outcomes, measured by response rate and survival. In the effort to overcome these issues, the development of “multifunctional” nanoparticles has been gaining great interest as a promising approach to integrate additional capabilities like targeting and image contrast enhancement [11]. However, additional functionality leads to a more complicated synthetic process, more complex pharmacological behavior, and also further regulatory hurdles. Consequently, there is an ongoing debate regarding the trade-off between additional functionality and complexity [108]. Partly because of this potential complication, the motivation to further invest in active targeting will likely

pose to the clinical benefits observed with the first generation of targeted nanoparticles that have reached clinical trials.

Efficiently generating targeted nanocarriers and translating them into clinical practice will imply the development of new clinically relevant models addressing several cancer biology aspects. In particular, the extensive tumor cell heterogeneity within a tumor mass and between primary tumors and metastasis has profound clinical implications and challenges the current design of active targeting nanoparticles for treating solid tumors. Phenotypic and functional differences arise among cancer cells within the same tumor as a result of genetic and epigenetic changes, environmental factors, and cancer cell plasticity [109]. Also, we know that the tumor microenvironment is composed of a complex variety of cell populations such as cancer associated fibroblasts, immune cells, endothelial cells, and stem cells as well as extra cellular matrix proteins. Aside from the need to develop a deep understanding of cancer biology, we envisage a future requiring the application of longitudinal biomarker measurements in the clinical setting to: i) map cellular and molecular heterogeneity within solid malignancies; ii) define a clear correlation between a specific molecular marker and a particular clinical outcome; iii) prevent drug resistance caused by clonal evolution [50]. Given that active targeting nanoparticles are supposed to be effective only to a specific subtype of tumors with a biomarker overexpression, reliable approaches for detection of biomarker-positive tumors are highly required for selection of patients who could benefit from such treatments. It has to be mentioned that in recent years cancer research has been intensively improved following the development of the cancer immunotherapy (that was awarded with the 2018 Nobel Prize in medicine to James P. Allison and Tasuku Honjo). Nanoparticles engineering has evolved in parallel and several enhancements of cancer immunotherapy has been reached so far thanks to the use of innovative biomaterials and delivery systems [110,111]. In particular, there are several evidences that the integration of nanotechnology and cancer immunotherapy can resolve some important issues such as the low response rate and the metastatic tumor targeting through the induction of the abscopal effect [112,113]. In our opinion, this field will continue to evolve rapidly and we believe

that the more preclinical advanced active targeted nanoparticles will soon be ready for clinical translation, in particular artificial biomimetic exosomes. The recent research on extracellular vesicles, that includes artificial biomimetic exosomes, was so intense that in the year 2018 the International Society of Extracellular Vesicles [114] needed to published a new position statement to better define them and regulate their use. In the cancer field, the main advantage of using artificial biomimetic exosomes relies on the high biocompatibility, on the adaptability to specific applications and on the high immune-tolerance (with the opportunity to prepare them from autologous cells for personalized nanomedicine). We believe that these features make them one of the most promising innovative drug delivery system for improving cancer therapy and we expect to observe important results from the first clinical trial, sponsored by the M.D. Anderson Cancer Center (NCT03608631, Phase I, not yet recruiting), that will treat with iExosomes patients affected by metastatic pancreatic cancer with $KRAS^{G12D}$ mutation.

Nonetheless, it is still unclear to what extent intratumoral biological barriers are hampering nanoparticles targeting to cancer cells. For example, despite the expectation that trastuzumab would facilitate specific delivery of nanoparticle to diseased cells *in vivo*, recent findings showed that the majority of the intravenously injected trastuzumab-coated gold nanoparticles were either trapped in the extracellular matrix or taken up by perivascular tumor associated macrophages (TAMs), only 14 out of 1 million reaching the cancer cells within solid tumor in a preclinical model [115]. Thus, a need exists to further investigate with a quantitative approach to the intratumoral fate of nanomaterials to correlate it to therapeutic efficacy. A complete picture of intratumoral barriers would assist in defining more effective strategies to improve the clinical translation of actively targeted nanoparticles.

It is finally important to consider that nanotechnology based on active targeting hold promises for improving the rapidly expanding field of genetic medicine. More than 100 investigational new drug applications (INDs) were filed last year for gene-therapy products [116], leading to advancement on the design engineering of the biotechnology platforms used for gene-therapy. Noteworthy is the

clinical performance achieved by Rexin-G®, the world's first tumor-targeted injectable gene therapy vector, which combines the enhanced selectivity for the tumor microenvironment and the toxicity of the genetic payload. The evidence that the gene delivery function of Rexin-G® remains active as it accumulate inside metastatic lesions within sentinel lymph nodes, and does not interfere with but appears to work in concert with the immune system, support the potentiality of future cancer vaccinations *in situ*, using this targeted gene delivery system bearing an immunomodulatory cytokine gene [117].

Figure 1. Schematic representation of the expression of TfR1 in normal and tumor cells together with the cellular iron homeostasis. Normal cell (**1**) The iron intake occurs when the complex Fe^{3+}-Tf-TfR1 is endocyted in the cytoplasm inside clathrin vesicles and it is regulated by intracellular iron levels. (**2**) Fe^{3+} disassociates from Tf-TfR1 complex, Fe^{3+} is then reduced to Fe^{2+} and transported in the cytoplasm by DMT1. (**3**) Fe^{2+} reachs intracellular compartments where it is needed. (**4**) The excess of iron is released in the extracellular environment through ferroportin. (**5**) In the nucleous iron participates in the synthesis and repair of DNA and in cell cycle. (**6**) TfR1 is recycled on the cell surface and (**7**) apoTf (transferrin without Fe^{3+}) is released externally. **Tumoral cell** (**8**) In response to inflammation, hypoxia, oxidative stress and mutations of the regulator genes, an increased expression of TfR1 occur. (**9**) Iron accumulates in the cytoplasm and upregulates the TfRC transcription. (**10**) Under the same conditions TfR1 mRNA translation is increased. (**11**) The efflux of iron is impaired since the expression of ferroportin is downregulated and its degradation is increased. (**12**) A large number of TfR1 receptors are exposed on the cell membrane. (**13**) TfR1 targeting by nanoparticles provides the potential to allow selected delivery of therapeutic agents to the tumor tissue.

TfR: Transferrin receptor; TfRC Transferrin receptor gene; DMT1: divalent metal ion transporter 1

Figure 2. **Role of folate receptor α (FRα) in actively targeted cancer nanomedicine**. (**A**) Tissue distribution of FRα is primarily confined to a limited number of polarized epithelia, which expression localized only at the apical surface of polarized cells, preventing exposure to the circulation. Upon tumorigenesis, intercellular junctions are lost and FRα loses its polarized cellular location, thus becoming positioned on the entire tumor cell surface. This process renders FRα accessible to actively targeted nanoparticles in blood circulation. Once a folate targeted nanoparticle is bound to FRα, it is internalized into the cell via the FR-mediated endocytic pathway. (**B**) FRα targeting by nanoparticles provides the potential to allow not only selected delivery of

therapeutic agents to the tumor tissue, but also tumor detection and intraoperative imaging. Also, theranostic nanoparticles enable simultaneous cancer imaging and therapy.

Figure 3. Actively targeted nanocarriers currently under clinical development as cancer drug delivery systems. Schematic representation of the targeting and therapeutic approaches along with brand names.

EGFR: Epidermal growth factor receptor; TfR: Transferrin receptor; XC proteins: Exposed collagenous proteins; PSMA: Prostate-specific membrane antigen; HER2: human epidermal growth factor receptor 2.

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Nanoparticles	Chemical-physical parameters	Tumor models	Targets	Cargos	Ref.
Traditional					
Liposomes	size distribution approximatively 100 nm; zeta-potential -24.3±1.3 mV	Pancreatic tumor	Transferrin	HIF-1alpha inhibitor and deferoxamine	[25]
Liposomes	size distribution 173.1±3.51; zeta potential -26.07±0.32 mV	Osteosarcoma	Alendronate (bone-targeting moiety) and anti-CD44 dual-targeting polymer	doxorubicin	[26]
Polymeric nanoparticles pH-sensitive	size distribution approximatively 200 nm	Breast cancer	RGD	copper chelator	[27]
Polymeric fluorescent nanoparticles	size distribution average 28 nm; MW 1243.550	Esophageal cancer	RGD	epirubicin	[28]
PLGA polymeric nanoparticles	size distribution average 201.4 nm; zeta-potential -8.63 mV	Breast cancer	Folic acid	docetaxel	[29]
Micelles	hydrodynamic radius 41.8 nm; MW $1.63x10^6$ g/mol	Multiple cancers	Anti-EGFR (by EgA1 nanobody)	doxorubicin	[30]
Mesoporous silica nanoparticles	size distribution 159±50 nm; surface charge -17.94±1.89 mV	Breast and prostate cancers	Triptorelin (agonist of gonadotropin-releasing hormone, GnRH)	doxorubicin	[31]
Gold nanoparticles	size distribution approximatively 3 nm	Acute myeloid leukaemia	Anti-Tim-3	rapamycin	[32]
Gold nanoparticles	size distribution 23±3 nm	Prostate cancer	PSMA-1 (prostate-specific membrane antigen)	Pc4 (fluorescent photodynamic therapy drug)	[33]
Iron oxide nanoparticles	size distribution approximatively 46 nm; magnetization value 58 emu/g	Lung cancer	Anti-EGFR	none (used for MRI and magnetic resonance-guided focused ultrasound surgery)	[34]
Iron oxide nanoparticles	size distribution 296 nm; zeta-potential +4.3±3 mV	Colon cancer	Folic acid	doxorubicin	[35]
Dendrimers	size distribution approximatively 13 nm; zeta-potential +4.8 ± 1.35 mV	Non-small cell lung cancer	Biotin	paclitaxel	[36]
Dendrimers	size distribution 50.74±26 nm; zeta-potential -5.9±0.35 mV	Liver cancer	iRGD-Cyp	paclitaxel	[37]
Carbon nanotubes	size distribution by TEM 20-30 nm; zeta-potential +20.4 ± 1.1 mV	Cervical cancer	Folic acid	doxorubicin	[38]
Innovative					
Nanoheterostructures (NHS) PD-L1-AuNP-DOX	size distribution 40±3.1 nm; surface charge -11.1±2.7 mV	Colorectal cancer	anti-PD-L1	doxorubicin	[39]
Biomimetics $CD47^+$ exosomes (iExosomes) from human skin fibroblasts	size distribution approximatively 100 nm	Pancreatic cancer	$KRAS^{G12D}$	siRNA	[40]
Biomimetics exosomes (SMART-Exos) from HEK293 cells	size distribution pick100 nm; zeta-potential -25±4.1 mV	Triple negative breast cancer (TNBC)	anti-CD3/anti-EGFR simultaneous target	None (used for cancer immunotherapy)	[41]
Biomimetic gold nanoparticles functionalized with TEV (tumor cell-derived extracellular vesicles) from 4T1 cells	size distribution average 100 nm	Brest cancer	Cell mimicking entry	anti-miR-21	[42]

Table 1. Examples of the most recent traditional and innovative nanoparticles for cancer active targeting preclinical research.

	TfR-targeted nanoparticles	Characteristics	Advantages on TfR targeting
	Tf/OX26-R17217 NHS PEG-MAL-5000 LOPARAMIDE HAS NPs	Human albumine serum nanoparticles loaded with loparamide and conjugated with Tf ligand or mAbs (OX26 or r17217)	Delivery of loparamide in the brain crossing the blood brain barrier [73]
Preclinical studies	**TMZ-mAb-PLGA NPs**	PLGA Resomer® RG503H loaded with temozolomide (TMZ) and conjugated with OX26 mAb Size 200 nm Net negative charge	Physicochemical features for brain delivery Great TMZ encapsulation efficiency OX26 mAb recognizes and links TfR Increase of the delivery into glioblastoma cancer cells Improvement of the anti-tumoral activity of TMZ [74]
	Tf-Ru-NPs	Photothermal agent-Ruthenium (Ru) polyvinylpyrrolidone (PVP) nanoparticles and conjugated with transferrin ligand Size distribution about 70 nm Low toxic 82,2% cell viability at concentration 200ug/ml	Selective cellular uptake receptor- mediated Increase of intracellular nanoparticles and improve photothermal therapy (PTT) Excellent capability to kill A549 cancer cells reducing side effects *in vitro* Ensure highly efficient *in vivo* PTT under NIR irradiation with 100% tumor elimination [75]
	Tx-NPs-Tf	Poly (D,L-lactide-co-glycolide) (PLGA), Cremophor_EL, zinctetrafluroborate hydrate, conjugated with holo-transferrin Size 220 nm Negative surface charge	Uptake 3-fold more than unconjugated NPs in PC3 prostate cancer cells Anti-proliferative effect and high toxicity using lower doses of PTX. *In vivo* a complete tumour regression and a better survival rate. [76]
	Tf-PTX-PLGA-NPs	PLGA nanoparticles loaded with Paclitaxel (PTX) and conjugated with transferrin Size about 170 nm	Higher concentration of PTX in male Sprague–Dawley rats bearing subcutaneous C6 glioma [77]
	Tf-MM-NPs	Dextran T40-covered polylactide (PLA) nanoparticles loaded with monomyristin and conjugated with Tf Size 187 nm	High efficiency of MM delivery into HeLa cells Elevated cytotoxicity against HeLa employing lower concentrations of encapsulated MM. [78]
	Tf-NPs	Polyethylene glycol (PEG)ylated liposomal nanoparticles (DSPE-PEG_{2K})loaded with TMZ+JQ1 and functionalized with Tf Size 137nm	Crossing of the Blood Brain Barrier High accumulation and retention in brains of mouse models of GBM Efficient delivery of dual combinations therapies anti-tumoral drugs Increased survival. Low systemic drug toxicity Translation of this nanoscale platform in human clinical trials [79]
Clinical studies	**SGT-53**	Cationic immunolipoplex complex with TfR scFv attached to the surface loaded with plasmid DNA coding p53 wild type gene Size 100 nm	Treatment solid tumors in combination with Docetaxel Good safety profile at therapeutic doses in a Phase 1b study [80]
	CALAA-01	Linear β-cyclodextrin-based polymer (CDP)-adamantane -polyethylene glycol (PEG-AD) formulation conjugated with human Tf ligand loaded with siRNA Size 70 nm	Stability in biological fluids Well tolerated in multi-dosing studies in non-human primates Intracellular localization Accumulation and retention in solid tumors Specific gene inhibition by activating siRNA machinery [81,82]

Table 2. Representative selection of nanocarriers targeted to TfRs highly expressed in cancers cells.

Nanoparticles	Therapeutic cargos	Comments
Human serum albumin nanoparticle	Cabazitaxel (CTX)	Preclinical studies have shown FA conjugation to improve safety and tolerability of CTX while preserving drug potency. The accumulation of FA-NPs-CTX at the tumor site was significantly increased, thereby ensuring a remarkable tumor inhibition [87].
Silver sulfide@mesoporous silica core-shell nanoparticle	Doxorubicin and survivin antisense oligonucleotide	This multifunctional nanoplatform for targeted image-guided treatment of tumor integrates fluorescence imaging/chemotherapy/ thermotherapy/gene therapy. The targeting ligand on surface of nanoparticle was an effective way to increase probe uptake by receptor-mediated endocytosis [88].
Annonaceous acetogenins nanosuspensions prepared using DSPE-PEG-FA and soybean lecithin as stabilizers (FA-PEG-ACGs-NSps)	Annonaceous acetogenins (ACGs)	Preclinical studies have shown that FA-PEG-ACGs-NSps could lead encapsulated drug to the tumor site, promote FR-mediated endocytosis upon binding to FR-positive cells, and circumvent the ACGs's poor solubility. The study demonstrate an overall improved antitumor efficacy, whilst also minimizing off-target toxicity [89].
Liposomes	Antisense oligonucleotide, verteporfin, gold nanoparticles and doxorubicin	These X-ray-triggerable liposomes incorporating gold nanoparticles and photosensitizer verteporfin possess versatile characteristics. They have the potential to attain synergistic effects of deep tissue photodynamic therapy and chemotherapy delivered via X-ray radiation [90].
Lipid-polymer hybrid nanoparticles	Vincristine	These nanoparticles showed improved therapeutic effect on an *in vivo* lymphoma animal model, and also reduced the systemic toxicity [91].
Polymer-coated gold/graphene hybrid nanoparticles	Paclitaxel	*In vitro* tests showed that these hybrid nanoparticles enabled to achieve simultaneously an excellent time-dependent photothermal effect (provided by gold/graphene composite) and a suitable chemotherapy (according to loading of paclitaxel) of folate receptor-positive cancer cells [92].
Wormlike mesoporous silica nanocarriers decorated with extremely small iron oxide nanoparticles and gold nanoparticles	Doxorubicin	This integrated drug delivery platform exhibited a MRI/CT dual imaging property with an enhanced specific cellular uptake toward cells with folate receptor.
Gold nanoclusters	Cisplatin	This theranostic nanoplatform for fluorescence tumor imaging and cancer therapy could efficiently inhibit the growth of the primary tumor and suppress the metastasis of the cancer cells to the lung in a orthotopically implanted 4T1 breast tumor model [93].

Table 3. Representative selection of folate-targeted nanocarriers from a vast field of conjugates that employ a folate ligand for active cancer targeting.

Study title	Target	ClinicalTrials.gov identifier (Phase)	Nanoparticle structure	Ref.
Phase I Study of TENPA in Advanced Solid Can	Not specified	NCT02979392	Paclitaxel is encapsulated in a thermodynamically stable core-shell structure composed of a serum protein and a targeting moiety assembled by non-covalent interactions.	
MesomiR 1: A Phase I Study of TargomiRs as 2nd or 3rd Line Treatment for Patients With Recurrent Malignant Pleural Mesothelioma and Advanced Non-Small Cell Lung Cancer	EGFR	NCT02369198	miR-15/16-derived microRNA mimics packaged in nanocells (formed through asymmetric cell division in bacteria) targeted with EGFR antibodies.	[102][105]
Safety Study of CALAA-01 to Treat Solid Tumor Cancers	Transferrin receptor	NCT00689065	siRNA (designed to reduce the expression of the M2 subunit of ribonucleotide reductase) is encapsulated in a self-assembly nanoparticle composed of a linear cationic cyclodextrin-based polymer modified with a terminal adamantane group (AD-PEG) and some AD-PEG conjugated to human transferrin.	[96]
Anti-EGFR Immunoliposomes in Solid Tumors	EGFR	NCT01702129	Doxorubicin-loaded anti-EGFR immunoliposomes were constructed by covalently linking antigen-binding fragments of the chimeric monoclonal antibody cetuximab to liposomes containing doxorubicin.	[106]
A Study to Evaluate the Safety, Tolerability and Immunogenicity of EGFR(V)-EDV-Dox in Subjects With Recurrent Glioblastoma Multiforme (GBM) (cerebral EDV)	EGFR	NCT02766699	Doxorubicin is encapsulated in bacterially derived minicell targeted with EGFR antibodies.	[104]
Safety and Efficacy Study Using Rexin-G for Sarcoma	Exposed collagenous (XC) proteins	NCT00505713	Tumor-targeted retroviral expession vector displaying a high-affinity collagen-binding motif derived from von Willebrand coagulation factor on its surface envelope and bearing a cytocidal dominant-negative cyclin G1 construct as its genetic payload.	[100]
Phase II study of combined temozolomide and SGT-53 for treatment of recurrent glioblastoma	Transferrin receptor	NCT02340156	Liposome nanoparticle carrying a plasmid encoding human wild-type p53 targeted with anti-transferrin receptor single-chain antibody (TfRscFV).	
A Phase 2 Study to Determine the Safety and Efficacy of BIND-014 (Docetaxel Nanoparticles for Injectable Suspension), Administered to Patients With Metastatic Castration-Resistant Prostate Cancer	Prostate-specific membrane antigen	NCT01812746	Polymeric nanoparticle targeting prostate-specific membrane antigen loaded with docetaxel.	[98]
A Phase 2 Study to Determine the Safety and Efficacy of BIND-014 (Docetaxel Nanoparticles for Injectable Suspension) as Second-line Therapy to Patients With Non-Small Cell Lung Cancer	Prostate-specific membrane antigen	NCT01792479	Polymeric nanoparticle targeting prostate-specific membrane antigen loaded with docetaxel.	[60]
A Study of BIND-014 Given to Patients With Advanced or Metastatic Cancer	Prostate-specific membrane antigen	NCT01300533	Polymeric nanoparticle targeting prostate-specific membrane antigen loaded with docetaxel.	
A Study of BIND-014 (Docetaxel Nanoparticles for Injectable Suspension) as Second-line Therapy for Patients With KRAS Positive or Squamous Cell Non-Small Cell Lung Cancer	Prostate-specific membrane antigen	NCT02283320	Polymeric nanoparticle targeting prostate-specific membrane antigen loaded with docetaxel.	
Safety and Pharmacokinetic Study of MM-302 in Patients With Advanced Breast Cancer	HER2	NCT01304797	HER2-targeted PEGylated liposomal doxorubicin	[103][107]

Table 4. Registered clinical studies using actively targeted nanoparticles in cancer drug delivery.

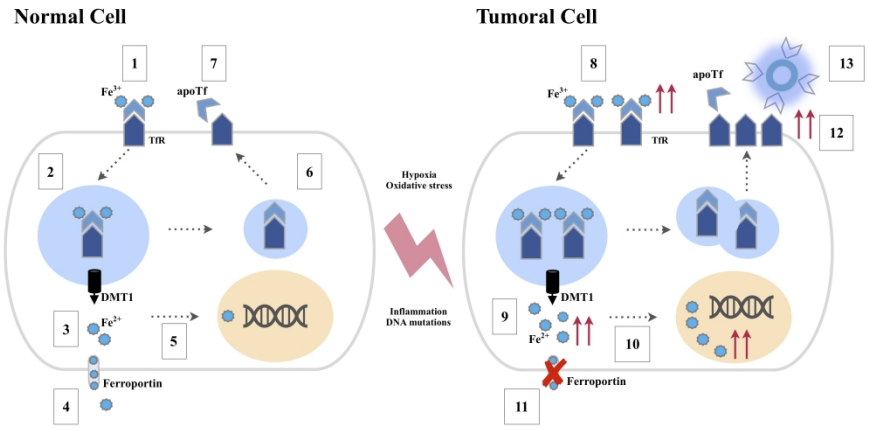

Figure 1

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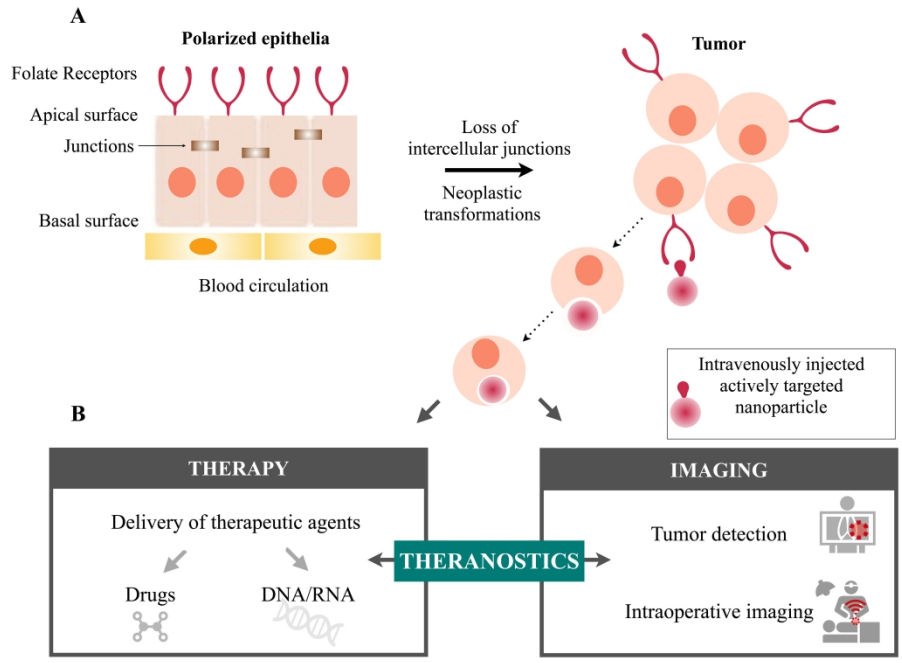
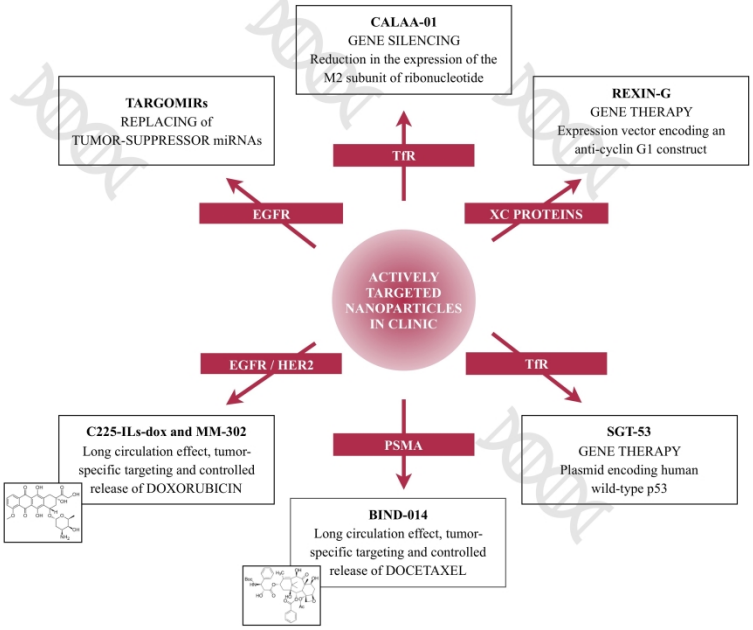

A
Polarized epithelia
Folate Receptors
Apical surface
Junctions
Basal surface
Blood circulation
Loss of intercellular junctions
Neoplastic transformations
Tumor
Intravenously injected actively targeted nanoparticle
B
THERAPY
Delivery of therapeutic agents
Drugs
DNA/RNA
THERANOSTICS
IMAGING
Tumor detection
Intraoperative imaging

Figure 2



361x270mm (300 x 300 DPI)