


Review

Nucleic Acid Aptamer-Guided Cancer Therapeutics and Diagnostics: the Next Generation of Cancer Medicine

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Abstract

Conventional anticancer therapies, such as chemo- and/or radio-therapy are often unable to completely eradicate cancers due to abnormal tumor microenvironment, as well as increased drug/radiation resistance. More effective therapeutic strategies for overcoming these obstacles are urgently in demand. Aptamers, as chemical antibodies that bind to targets with high affinity and specificity, are a promising new and novel agent for both cancer diagnostic and therapeutic applications. Aptamer-based cancer cell targeting facilitates the development of active targeting in which aptamer-mediated drug delivery could provide promising anticancer outcomes. This review is to update the current progress of aptamer-based cancer diagnosis and aptamer-mediated active targeting for cancer therapy *in vivo*, exploring the potential of this novel form of targeted cancer therapy.

Key words: aptamer, cancer therapy, active targeting, cancer stem cell, chemoresistance, cancer theranostic

Introduction

The success of cancer chemotherapy depends on enough delivery of therapeutic drugs to tumors while sparing normal tissues. Inadequate amounts of drug reaching cancer cells after initial treatment is responsible for an increase in drug resistance and tumor recurrence, which are due to an outgrowth of residual tumor cells [1]. Factors such as abnormality in tumor microenvironment and tumor cell heterogeneity contribute to the failure of cancer treatment. The abnormal tumor physiology, such as vasculature changes, introduces a set of delivery challenges that limit drug

transport to original and metastatic tumor sites, and in return promotes systematic drug resistance [2]. Aptamers are short chemically synthesised single-stranded RNA or DNA oligonucleotides that specifically bind to molecular targets with high affinity and specificity. They can be further chemically modified to acquire desirable attributes for clinical applications [3]. A wide range of diagnostic agents or chemotherapeutic agents can be linked to aptamers, via physical or chemical conjugation, to enhance cancer diagnosis and treatment efficacies. This review

will provide an overview of obstacles that therapeutic agents face and discuss the latest advances in aptamer-based cancer diagnostics and therapy.

Pathophysiology challenges for drug transport to solid tumors

During the course of pharmacological treatment of cancer, delivery and metabolism of a chemotherapeutic agent is a complex journey starting from the site of administration and ending where the tumor cell is located. The five main factors involved in this journey are discussed in the following sections [4, 5].

Abnormal blood and lymphatic vessels

Efficient drug delivery to solid tumors relies on good blood circulation in the tumor, followed by drug transportation through vascular walls [4, 5]. In the normal angiogenesis system, there is a balance between new blood vessel growth and shrinking of the old one under the influence of antiangiogenic factors [6, 7]. In contrast, tumor angiogenesis is characterised by uncontrolled overproduction of new blood vessels that are inhomogeneous in size and density, thus hampering fluid perfusion and drug transportation and resulting in further drug resistance, tumor mutagenesis, metastases and/or recurrence [8-10]. The lymphatic system functions to return interstitial fluid to the blood circulation and to clear macromolecules as well as detached tumor cells [11, 12]. The impaired lymphatic drainage is a prominent characteristic of solid tumors and this, coupled with a leaky tumor vasculature, results in an enhanced accumulation and retention of molecules with a molecular mass of 40 kDa or higher in the tumor, an important phenomenon known as the enhanced permeability and retention (EPR) effect [8, 13, 14]. The compressed lymphatic vessels lose their function of returning interstitial fluid, resulting in fluid retention and elevation of interstitial fluid pressure (IFP), generating another barrier for drug transport [8, 15].

Elevated interstitial fluid pressure (IFP)

Interstitial fluid is the extracellular solution that bathes and surrounds most tissues, excluding the extravasation fluid from the lymph and blood vessels [16]. The interstitial fluid mediates the exchange of oxygen, nutrients and waste products between cells and their surroundings through the interstitial space among capillaries, blood vessels and the lymphatic system [17]. IFP is the hydrostatic and colloid osmotic pressures exerted by the free interstitial fluid. In contrast to normal interstitium, the IFP is often elevated in solid tumors partly because of low lymphatic drainage and leaky tumor vessels [16-19]. The elevated IFP is a major obstacle for anticancer drug

transport [20]. Many factors can lead to an increased IFP, including individual or combined effects of hyper-permeable blood vessels and a defective lymphatic system [21-23]. Chemotherapeutic drugs are transported and distributed through the tumor interstitial space via diffusion and convection, for small and large molecule transport, respectively [24-26]. Drug diffusion depends on diffusivity and concentration gradients, while convection of large molecules relies on hydraulic conductivity and pressure differences [27]. High molecular weight drugs mainly travel to the interstitial space by convection, but the uniform IFP and negligible fluid pressure gradients weaken the convection power, leaving diffusion largely responsible for drug transport in solid tumors [28, 29]. Increased IFP decreases vascular and capillary functions and limits drug exposure, distribution and uptake by tumor cells, resulting in the failure of anticancer therapy [4, 30].

Dense interstitial structure and extracellular matrix

Extracellular matrices in healthy tissue serve many functions, including maintaining cellular homeostasis, sequestering tissues or cellular growth factors from one to another, and eliciting protease activities for regulating cellular functions [31]. The abnormal extracellular matrices in the tumor are largely composed of structural macromolecules (collagen) and space-filling proteins (polysaccharides) that make the tumor interstitium a highly viscous space [31, 32]. This viscoelastic and tortuous area forms a major barrier for effective drug transport [30]. Components such as collagen, glycosaminoglycan and fibroblasts are responsible for the lower hydraulic conductivity and increase of interstitium tension, resulting in a poorer convection and increased flow resistance [4, 5]. In addition, the dense interstitial matrices mediate solid stress and compress blood and lymphatic vessels, leading to further restrictions on drug distribution.

High tumor cell density

High cell density is also responsible for the increase in IFP, compression of blood vessels, and extracellular matrix that lead to a dense and tortuous tumor microenvironment [9, 33]. In contrast, low cell density along with larger interstitial spaces facilitates more effective drug transport to their targets.

Tumor acidic environment

The leaky tumor vessel is inadequate to supply enough nutrients, resulting in a reduction in the transport of oxygen to tumor cells [34, 35]. Together with hypoxia, the nutrient-deprived niche provides a physiological-associated pressure stimulating the

development of malignant tumor cells as well as resistance to chemotherapy [36-39]. The low pH (pH 5.7-7.4) in the tumor can naturally induce cell death, but cells that live in such conditions are hard to be killed, even with drug treatment, contributing to a failure of cancer therapies and disease progression [36-39].

Aptamer-mediated drug delivery for targeted cancer therapy

Aptamer

Aptamers, nucleic acid-based ligands, are small single-stranded DNA or RNA oligonucleotides that are produced *in vitro* via a process known as systematic evolution of ligands by exponential enrichment (SELEX) [40, 41] (Fig. 1). Isolation of aptamers specific for the target of interest can be achieved via SELEX

from a large pool ($> 1 \times 10^{14}$) of single-stranded oligonucleotides with random sequences [42, 43]. After the incubation of the random aptamer pool with the target followed by the removal of non-binding aptamers, the bound aptamer species are recovered. These recovered nucleic acid sequences are amplified with PCR (in the case of DNA aptamer) or RT-PCR (in the case of RNA aptamers). An enriched pool of potential binders generated from PCR amplification is used in the subsequent selection rounds (typically 6-10 rounds). At the last round of SELEX, the resulting highly enriched pool of aptamers are cloned or subjected to next generation sequencing followed by further characterization and engineering of individual cloned aptamers (Fig. 1). In addition to selection against a purified or highly enriched target molecule, one can perform SELEX using live cells and even in a whole mouse to isolate cell- or organ-specific aptamers against a specific type of cells or an organ/tissue [44, 45]. Therefore, the power of SELEX enables one to generate specific aptamers against an inorganic or organic molecule, a protein, a cell and even an organ of interest.

Aptamers fold into tertiary conformations and bind to their targets through shape complementarity at the aptamer-target interface [46]. An aptamer binds to a protein can modulate protein functions by interfering with protein interaction with natural partners. Besides to binding to small organic and inorganic molecules, aptamers have the unique ability to recognize and bind to large targets, such as proteins, whole cells or even organs [47-50]. Similar to antibodies, aptamers gain entrance to target cells via receptor-mediated endocytosis upon binding to cell surface ligands [51, 52]. In addition, the *in vitro* generation of aptamers via SELEX confers a low-cost ad-

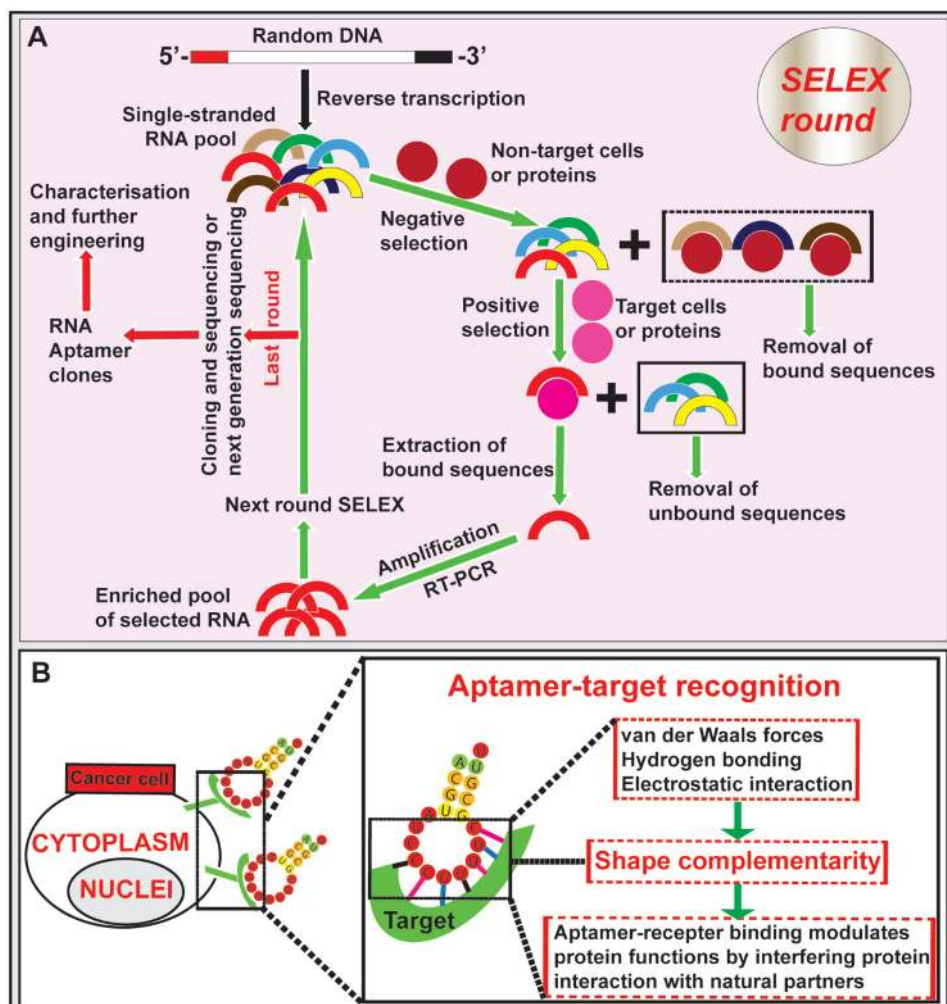


Figure 1. Isolation of aptamer and aptamer-target cell interaction. (A) Schematic illustration of RNA aptamer generation by SELEX. Negative control cells or proteins are incubated with a large pool ($> 1 \times 10^{14}$) of single-stranded RNA oligonucleotides with random sequences, followed by removal of bound RNAs. The unbound sequences are then subjected to positive selection by incubating with specific targets of cells or proteins. The bound aptamers are eluted and amplified by RT-PCR, followed by new SELEX rounds. After the last round of selection (typically 6-10 round), the remaining RNA aptamers are converted to DNA and subjected to next generation sequencing or conventional cloning followed by characterization and further engineering. (B) Schematic diagram depicting the aptamer-target interactions. Aptamers bind to targets at the cell surface through shape complementarity involving non-covalent bonds.

vantage over the long and arduous development process of antibodies [51, 53-55]. One important advantage of aptamers over antibodies is that, once selected, they can be chemically synthesised instead of being produced in animals or cultured mammalian cells, thus simplifying the production of therapeutic grade materials, which represents a key advantage for commercial development [56, 57]. Importantly, aptamers can penetrate into tumor cores much more efficiently than antibodies due to their ~20-25-fold smaller sizes compared with full sized monoclonal antibodies [58-60]. Given that the nucleic acid aptamers function *in vivo* through the blood plasma, several limitations of aptamers should be considered. Being polynucleotides, nucleic acid aptamers are naturally susceptible to enzymes degradation by exo- and/or endo-nucleases, leading to a reduced *in vivo* circulatory half-life. This drawback can be alleviated by introducing backbone or side chain chemical modifications to aptamers, incorporating unnatural nucleotide bases (locked and unlocked nucleic acids) and capping the aptamer ends, thus minimizing the susceptibility to endonuclease and exonuclease attack [61-63]. Short blood residence time is another challenge with *in vivo* aptamer applications, which is due to fast removal of aptamer from the circulation by renal filtration as most aptamers have a size smaller than the renal filtration threshold of 40 kDa [63]. To achieve desired serum half-life, aptamers can be engineered by conjugation with a terminal polyethylene glycol (PEG), although this may compromise the extent of tumor penetration [62, 64]. It is worth noting that post-SELEX modifications following the selection of aptamers may alter the 3-D structure of the aptamers, leading to the lost or altered binding affinity and specificity. Such risks can be prevented by using random aptamer pools containing modified nucleotides during the SELEX selection [63, 64]. In addition, the ability of aptamers to interact with cells may decrease due to repulsion of nucleic acids by negatively-charged cell membranes [49]. This can be refuted by increasing the binding affinity and specificity of aptamers toward their cell surface receptors to trigger receptor-mediated endocytosis [61].

Aptamer-guided active targeting system: overcoming pathophysiology barriers

The ultimate aim of anticancer research is to improve the rates of treatment response and overall survival in patients with cancer. To achieve this goal, a tumor targeting system that delivers therapeutic agents selectively to cancer cells is necessary [65]. Passive targeting based on EPR utilises the fundamental differences in the organisation of vasculatures between normal tissues and a tumor to achieve pref-

erential accumulation of nano-sized anti-tumor agents in tumors [66]. Based on the EPR effect, a passive tumor-targeting strategy has improved patient compliance and reduced adverse effects over the conventional free drug approach [67]. However, there have been heated debate on the existence of EPR and whether EPR plays a role in enhanced delivery of nano therapeutics in at least some types of tumors [66, 68]. Moreover, the clinical efficacy of nano-sized drugs guided by a passive targeting system is still far from optimal due to poor tumor penetration and specific drug uptake by cancer cells [67, 69]. To further improve treatment efficacy, an "active targeting" approach has been attracted huge attentions. The function of active targeting is to guide therapeutic agents with the aid of targeting ligands to tumor cells and promote their subsequent cellular entry through receptor-mediated endocytosis [70-72]. Development of novel molecular probes that bind to specific tumor biomarkers as actively guiding devices represents one of the major advances in the next generation of tumor therapeutics.

The goal of surface modification of drug carriers by tumor specific recognition molecules, such as antibodies, peptides and aptamers, is to enhance specific drug accumulation, internalization and retention in tumors via specific ligand-mediated interactions thus increasing the therapeutic index [73, 74]. Targeting specificity and drug delivering capacity are two important aspects that need to be considered in the development of an active targeting system [70]. Antibodies are the most commonly used target ligands due to their high specificity and wide availability [51, 52]. Recently, aptamers have emerged as an attractive alternative to antibodies as a ligand for active targeting of tumors (Fig. 2). Actively targeted nano-sized therapeutic is envisioned as a promising complementary strategy to passive targeting via EPR to further augment the efficiency of cancer nanomedicines. In this regard, effective delivery of drugs to tumors can be achieved via active targeting utilizing tumor-specific aptamers binding to their targets present on the surface of tumor cells. For instance, epithelial cell adhesion molecule (EpCAM) is expressed at low levels in normal epithelium, but is overexpressed (up to 1000-fold) in various solid tumors, including colon, hepatic and breast carcinomas, which relates to poor prognosis [49, 75]. Aptamer-guided active targeting enables the increased delivery of therapeutic agents to tumors as well as a reduction in toxicity and side effects by minimizing the exposure of normal tissues to the therapeutic agent. As an active targeting ligand, antibodies suffer from immunogenicity as even humanised antibodies may elicit immune responses in patients [57, 76, 77]. In contrast, being nucleic acids,

aptamers are generally non-immunogenic or low-immunogenic [78, 79]. The chemical synthesis of aptamers confers additional advantages to aptamers, such as low batch-to-batch variation, and they are easier to scale up production for large scale manufacturing with minimal risk of contamination of microorganisms and endotoxins [76, 80, 81]. Therefore, aptamers are being intensively investigated as specific

targeting moieties for cancer therapy. Aptamer-mediated active targeting for cancer therapy can be categorised into four groups: free aptamer as molecularly targeted agents, aptamer-drug conjugates, aptamer-nanoparticle-drug delivery system and aptamer-mediated cancer nucleic acid therapy (Fig. 3).

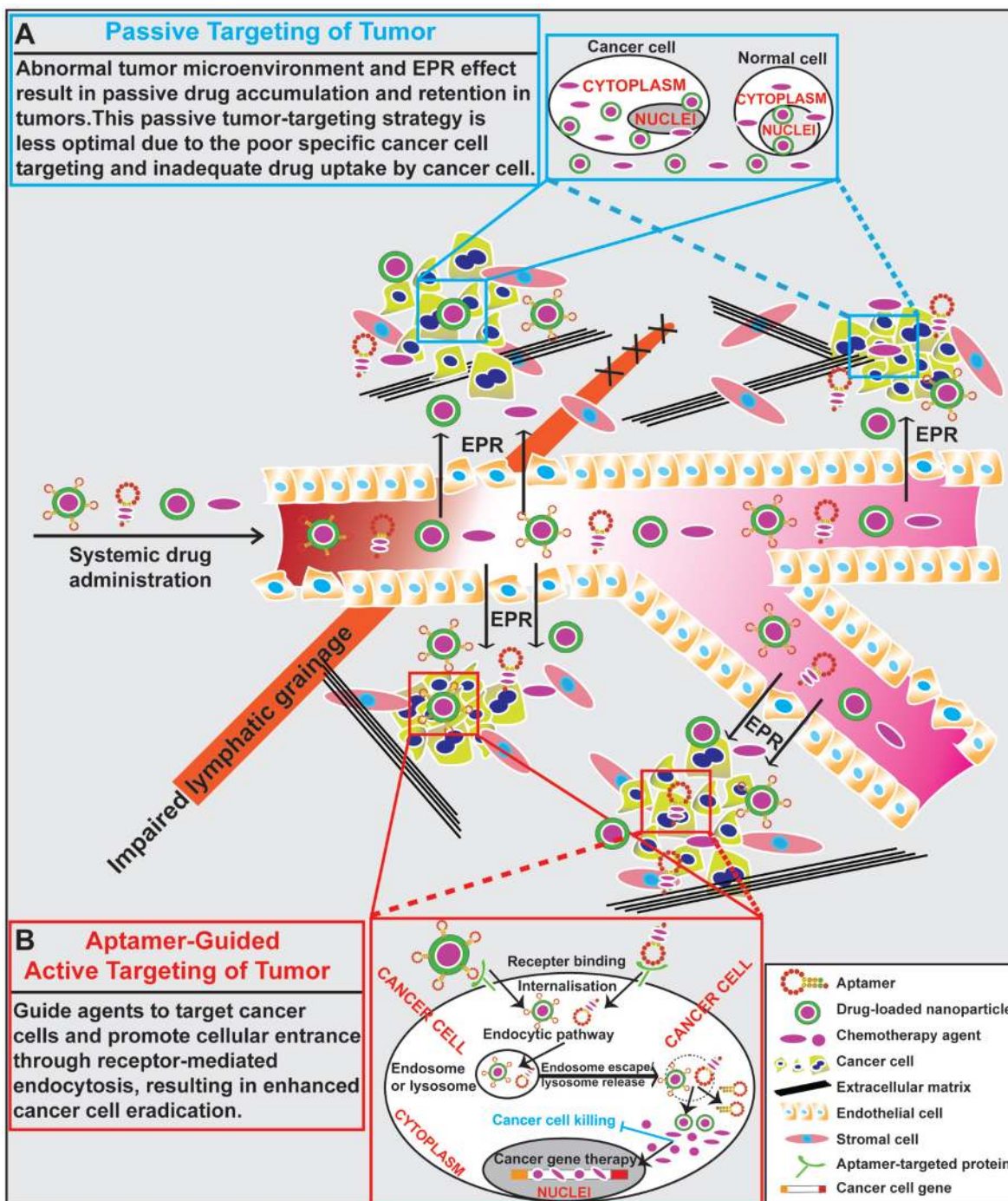


Figure 2. General scheme of drug delivery to tumors. (A) Schematic illustration of drug delivery via passive targeting. Abnormal tumor microenvironment and EPR effect result in passive drug accumulation and retention in tumors. This passive tumor-targeting strategy exhibits limitations of poor cancer cell targeting and inadequate drug uptake. (B) Schematic illustration of aptamer-guided active targeting of tumor. Aptamers undergo endocytosis after binding to cell surface targets, along with aptamer-conjugated therapeutic agents or drug-encapsulated nanoparticles. Following the entrance and escape from endosomes and/or lysosomes, the released therapeutic agents or small RNAs engage their cytoplasmic or nucleic targets to eliminate cancer cells.

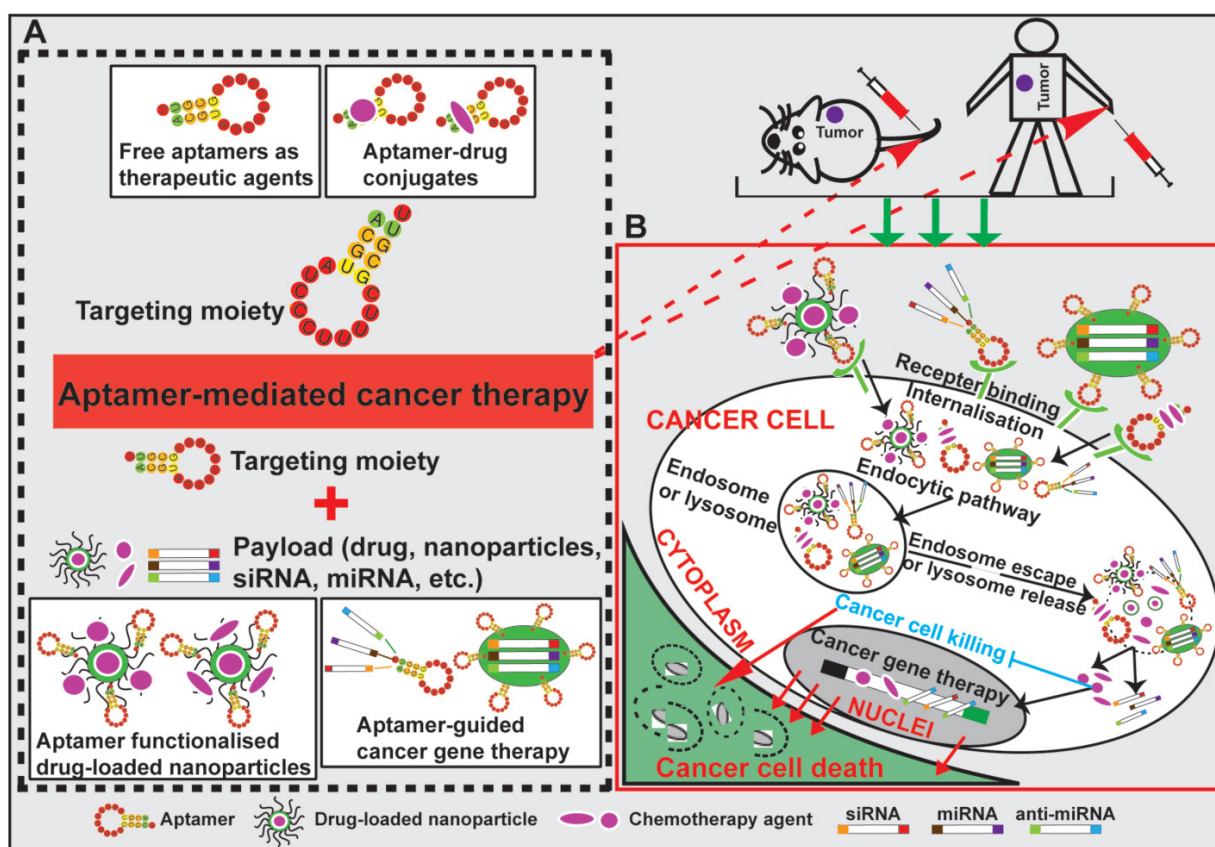


Figure 3. Schematic illustration of aptamer-mediated cancer therapy. Four approaches are presented: free aptamer as chemotherapeutic agents, aptamer–drug conjugates, aptamer–nanoparticle–drug delivery system and aptamer-guided cancer gene therapy. After specific binding of aptamers to their cell surface target, aptamer-conjugated therapeutic agents (including exogenous therapeutic nucleotides) or drug-encapsulated nanoparticles are internalised into cancer cells via receptor-mediated endocytosis. Subsequent escape from endosomes and/or lysosomes enables the released therapeutic agents to eradicate cancer cell.

Free Aptamer as molecularly targeted therapeutics

DNA aptamer targeting vimentin for tumor therapy

To study the anti-tumor activity of a DNA aptamer, Zamay and colleagues selected a DNA aptamer NAS-24 (80-nt in length) that targets vimentin and investigated its efficacy in inducing apoptosis of mouse ascites adenocarcinoma cells *in vivo* [82]. They utilized arabinogalactan (AG), a natural polysaccharide with low toxicity ($LD_{50} > 5\text{g/kg}$) and high biocompatibility, to enhance *in vivo* delivery of anti-vimentin aptamers [83, 84]. *In vivo* anti-tumor efficacy of AG-NAS-24 self-forming complex was evaluated using an Ehrlich ascites adenocarcinoma mouse model in which one million adenocarcinoma cells were transplanted into the abdominal cavity of imprinting control region male mice. Starting from day 4 post cell inoculation, mice were treated with AG-NAS-24 complex ($1.6\mu\text{g/kg}$), free AG or aptamer daily via intraperitoneal injection for 5 days. On day nine, following the 5-day treatment, there was a 5-fold higher suppression of carcinoma growth in mice

treated with AG-NAS-24 compared with those treated with free aptamer, free AG or saline. Aptamer delivered by AG caused ~2-fold more apoptosis in ascites tumor cells than that treated by saline or AG alone [82]. After intracellular delivery of AG-NAS-24 complex, aptamers may promote vimentin degradation which causes morphological changes and apoptosis in cancer cells with higher vimentin expression [82]. This study illustrates that intracellular delivery of NAS-24 aptamer could target vimentin and initiate signalling events leading to cellular apoptosis.

Aptamer-mediated tumor cell apoptosis and suppression of tumor progression

Myeloid-derived suppressor cells (MDSC) and tumor-associated macrophages (TAM) can enhance tumor angiogenesis, invasion, and limit the immunotherapeutic function of T-cells, resulting in further tumor aggression and metastasis [85-87]. In order to selectively target MDSCs and TAM activities for inhibiting the tumor progression, Roth *et al.* generated an RNA aptamer, cl.42 with 79 nucleotides, that specifically binds to IL-4 receptor- α (IL4Ra) with a $K_d = 14\text{ nM}$, which is a critical molecule for MDSC function [85]. In the IL4Ra $^{+/+}$ or IL4Ra $^{-/-}$ 4T1 breast can-

cer-bearing mice, cl.42 aptamer or a control aptamer (16 pmol/L/g) were administrated intravenously 3 times a week. In addition to a reduction of MDSCs and TAM expression *in vivo*, tumor progression was significantly inhibited in the IL4Ra^{+/+} tumor-bearing mice treated with the cl.42 aptamer. In contrast, no discernable anti-tumor effect was observed in mice bearing IL4Ra^{-/-} 4T1 tumors or those treated with the control aptamer. To better understand the effect of the anti-IL4Ra aptamer on MDSCs, Roth *et al.* isolated immortalized mesenchymal stem cells (MSC2) and MDSCs from IL4Ra^{+/+} tumor-bearing mice. Cell viability of MDSCs was analysed after treatment with cl.42 aptamer or control aptamer for 1-4 days. Compared to untreated or control-aptamer treated groups, the cl.42 aptamer treatment resulted in a 2-fold decrease in viability on day 1, and a 3-3-fold higher increase in apoptosis in MSC2 cells. More importantly, only the IL4Ra-specific aptamer (150 nM), but not the control irrelevant aptamer, triggers MDSC apoptosis and drastically reduced phosphoSTAT6 (pSTAT6) signalling which is known to play crucial roles in MDSC activation. This study suggests that aptamer-triggered apoptosis in MDSCs via blocking of the IL4Ra-STAT6 signalling pathway could be a promising strategy to arrest immune escape in cancer treatment. Recent studies which utilize free aptamers as molecularly targeted agents are summarized in Table 1.

Table 1. Application of free aptamers as cancer-targeted therapeutics

Aptamer	Target	Cancer	References
DNA aptamer	DNA-binding proteins	Ovarian cancer and breast cancer	Mern <i>et al.</i> 2010 [88, 89]
DNA aptamer	MCF-10TA1 cell	Breast cancer	Tan <i>et al.</i> 2011 [90]
DNA aptamer	VEGF165 protein	Human hepatocellular carcinoma	Yung <i>et al.</i> 2013[91]
DNA aptamer	AGE product	Melanoma	Yamagishi <i>et al.</i> 2014 [92]
RNA aptamer	CEA protein	Colorectal cancer	Lee <i>et al.</i> 2012 [93]
RNA aptamer	MDSC and TAM	Multiple carcinomas (colon, mammary, fibrosarcoma, melanoma)	Serafini <i>et al.</i> 2012 [85]

AGE, advanced glycation end; CEA, carcinoembryonic antigen; MDSC, myeloid-derived suppressor cells; TAM, tumor-associated macrophage; VEGF, vascular endothelial growth factor.

Aptamer-drug delivery system

Conventional therapeutic drugs often lead to severe adverse effects. Conjugating chemotherapeutic drugs to tumor-targeting aptamers can increase the drugs delivery to tumor cells while minimizing the exposure of non-target sites to the chemotherapy agents [94]. Doxorubicin (DOX) is one of the most potential anticancer agents ever developed and has been used for treatment of a wide range of liquid and solid cancers, including acute lymphoblastic leukemia

and malignant lymphomas, breast, ovarian, prostate, and bronchogenic carcinomas [95]. DOX can intercalate into genomic DNA, resulting in the disruption of DNA replication and apoptotic cell death [96]. However, its efficacy is impeded by dose-limiting cardiotoxicity, inspiring intense effort in transforming this free drug into a new and targeted DOX-delivery system [97]. Aptamers are known to form tertiary structures with short double stranded regions through intra-molecular base pairing [98], which is available for the intercalation of DOX to form a physical complex. In order to investigate the anticancer efficacy of aptamer-DOX conjugates, Tan's group developed two DNA aptamers sgc8c and TLS11a-GC that specifically bind to CCRF-CEM cells (T-cell acute lymphoblastic leukemia, T-cell ALL) and LH86 cells (a human hepatocellular carcinoma cell line), respectively [52, 99]. After intercalating DOX to the TLS11a-GC aptamer, they evaluated whether the aptamer-DOX conjugate could specifically kill their targets with a low toxicity towards non-target cells. Cell viability tests demonstrated that the aptamer-DOX conjugate exhibited high therapeutic potency similar to free DOX, but prevented the nonspecific uptake of membrane-permeable DOX to non-targeted cells. After the establishment of an *in vivo* liver cancer model, the tumor bearing NOD Cg-Prkdc (SCID) IL2 mice were treated with DOX and TLS11a-GC-DOX complex (2 mg/kg) via a tail vein injection on days 1, 2, 3, 4, 5, 9, 10 and killed on day 11. The TLS11a-GC-DOX complex had a more efficient anti-tumor effect (2-fold more decrease in volume) compared to free DOX, which indicated that TLS11a-GC-DOX conjugates increased the local DOX concentration in the tumors with the aid of a targeting aptamer. This aptamer-drug intercalation approach thus represents a promising tactic for targeted drug delivery to cancer cells. Recent examples of developing aptamer-drug conjugates as targeted therapeutics for cancer are summarized in Table 2.

Table 2. Application of aptamer-drug conjugations as cancer-targeted therapeutics

Aptamer	Target	Drug	Cancer	Research Group
DNA aptamer	CCRF-CEM cell	DOX	T-cell ALL	Tan <i>et al.</i> 2009 [100]
DNA aptamer	LH86 cell	DOX	Human hepatocellular carcinoma	Tan <i>et al.</i> 2012 [52]
DNA aptamer	MUC1 protein	DOX	Lung cancer and breast cancer	Yang <i>et al.</i> 2012 [101]
DNA aptamer	HER2 protein	DOX	Breast cancer	Yang <i>et al.</i> 2012 [102]
RNA aptamer	EpCAM protein	DOX	Retinoblastoma	Krishnakumar <i>et al.</i> 2012 [103]
RNA aptamer	EGFR protein	GEM	Pancreatic cancer	White <i>et al.</i> 2012 [104]

ALL: acute lymphoblastic leukemia; CCRF-CEM, T-acute lymphoblastic leukemia cell line; GEM, gemcitabine; LH86, human hepatocellular carcinoma cell line.

Aptamer-nanoparticle-drug delivery system

The past decade has witnessed promising advances in the synthesis and characterization of various nano-materials, which have been optimized for anti-cancer drug delivery vehicles [105]. The hybrid aptamer-nanoparticle system significantly enhanced

cancer-specific cytotoxicity both *in vitro* and *in vivo* [106, 107] (Fig. 4). Aptamer-guided nano therapeutics aid in circumventing pathophysiology barriers to enhance both the uptake and retention of drugs by tumor cells, leading to improved therapeutic efficacy [70, 108]. There are four major merits when nanoparticles are surface functionalized by aptamers for targeted cancer drug delivery [109]: 1) the specific recognition of aptamers to targets enables a targeted detection or binding to cancer cells; 2) the straightforward synthesis and chemical modification of aptamers can boost the translation of aptamers into clinical practice; 3) the biocompatible nanoparticle-loading ability enhances the intensity of analytical signal, leading to effective cell recognition, drug delivery and anticancer treatment, and 4) aptamers linking to the surface of nanoparticles facilitates internalisation and cellular uptake of drugs with the aid of aptamer-guided active targeting. Aptamers have been extensively used to functionalize nanomaterials for targeted drug delivery, which is briefly presented below.

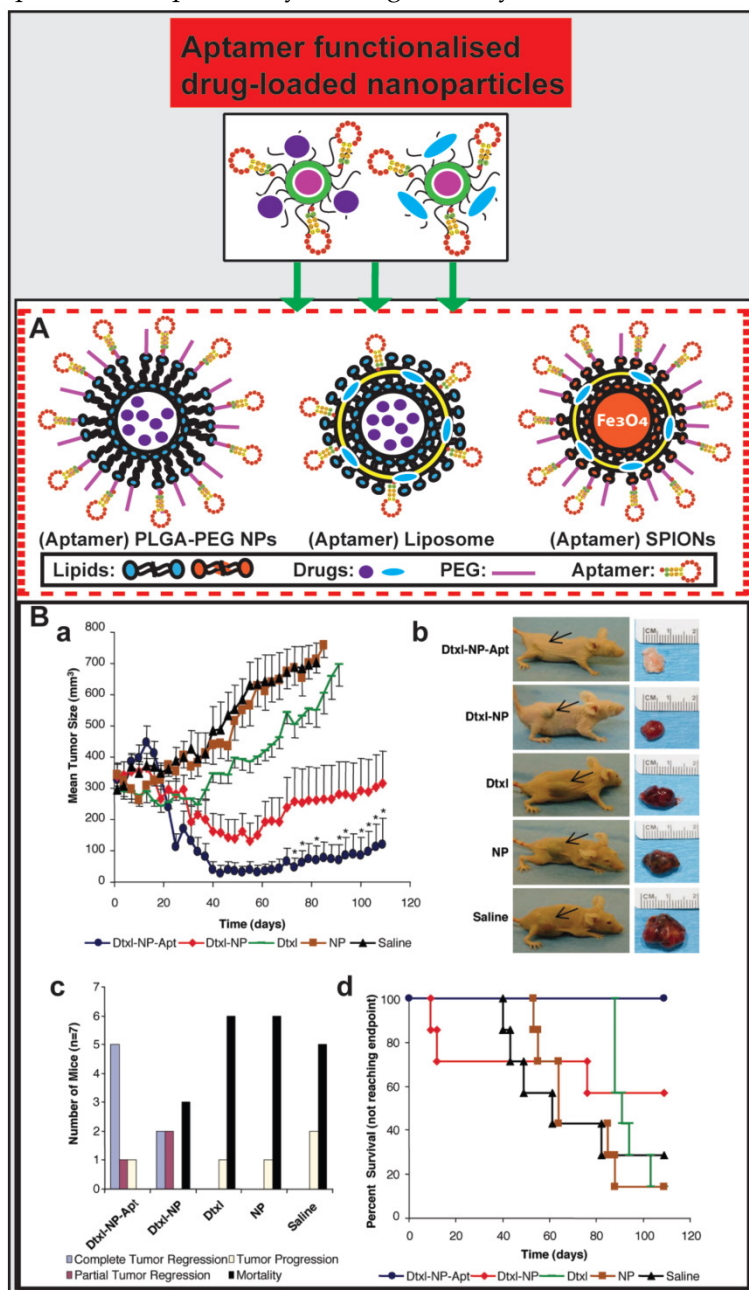


Figure 4. Aptamer-guided nano-drugs and their applications. (A) Schematic illustration of aptamer functionalised and drug-encapsulated nanoparticles. (B) Representative results from studies by Farokhzad *et al.* on A10 RNA aptamer functionalised Dtxl-encapsulated PLGA-PEG nanoparticles (Dtxl-NP-Apt) and anticancer efficacy *in vivo*. Even a single intratumoral administration of Dtxl-NP-Apt is significantly more efficient in tumor reduction as evident from the ~6.5-fold smaller tumor volume than that in mice treated with the controls (saline, NP, or Dtxl) (a and b) and improved survival (109 days vs. 73 days in Dtxl-NP group) (c and d). Reproduced from Ref. [113], Copyright (2006), with permission from National Academy of Sciences, U.S.A..

PLGA-PEG nanoparticles

Biodegradable lipid-PLGA-PEG nanoparticles take advantage of polymeric nanoparticles and have emerged as a robust drug delivery platform. They are composed of PLGA as a hydrophobic core for encapsulating drugs and a PEG shell which can prolong circulation half-life *in vivo* and link targeting ligands [110, 111]. PLGA-PEG nanoparticles conjugated with aptamers function via both active and passive targeting for anticancer drug delivery [26].

Farokhzad and co-workers encapsulated docetaxel (Dtxl) with PLGA-PEG nanoparticles that were functionalized with a 2-fluoropyrimidine modified RNA aptamer (A10) that binds to the prostate-specific membrane antigen (PSMA) [112, 113]. These Dtxl-encapsulated bioconjugates (Dtxl-NP-Apt) effectively targeted PSMA-expressing LNCaP prostate cancer cells and were taken up by these cells leading to significant enhancement of cellular toxicity *in vitro* compared with that of control nanoparticles lacking PSMA aptamers ($P < 0.004$) and control cells that do not express PSMA proteins. After the establishment of an LNCaP xenograft model in BALB/c nude mice, they treated the mice

with Dtxl-NP-Apt and other controls at a reported tolerated dose of 40 mg/kg Dtxl [114]. Surprisingly, even a single intratumoral administration of Dtxl-NP-Apt could achieve significant efficacy in tumor reduction (a 6.5-fold higher reduction in tumor volume than any of control treatment groups of saline, NP, or Dtxl), better survival time (109 days vs. 73 days in Dtxl-NP group) and decreased toxicity as compared with non-targeted NPs and controls (Fig. 4B). This study demonstrates the bioconjugation between PLGA-PEG nanoparticle and aptamer constitutes a promising strategy for future targeted cancer therapy.

AS1411, a 26-nucleotide DNA aptamer which specifically binds to the external domain of nucleolin, has been extensively used for glioma-targeted therapy [115], as nucleolin is a nuclear matrix protein over-expressed on the cell surface of several types of cancers, including lung cancer, glioblastoma (C6 cells) and pancreatic cancer. Chen *et al.* developed AS1411 aptamer functionalised PLGA-PEG nanoparticles encapsulated with Paclitaxel (PTX) (Apt-PTX-NPs) and evaluated the aptamer-guided anti-glioma efficacy of PTX [116]. Following the same dose administration, this AS1411-PTX-NPs showed a 2.3-fold and 5.4-fold increase in circulation half-life and AUC compared to free Taxol, respectively. Mice or rats bearing glioma xenografts were intravenously administered with Taxol, PTX-NPs, Apt-PTX-NPs (equivalent to PTX of 3 mg/kg) every 2 days for seven consecutive injections. Until the 20th day on which the animals were humanely killed, Apt-PTX-NPs treated mice exhibited a 1.7-fold higher tumor inhibition than those treated with PTX-NP, while the prolonged survival of tumor-bearing rats received Apt-PTX-NPs was observed compared with those treated with PTX-NP ($P < 0.05$) and Taxol ($P < 0.01$).

Liposomes

Liposomes have been successfully utilised as a very versatile drug delivery carrier. Various sizes and formulation methods of liposomes contribute to a wide range of options for drug delivery in tumor treatment [117, 118]. The liposome surface can easily be decorated with PEG, which can prolong the *in vivo* half-life in systemic circulation, resulting in the accumulation of chemotherapeutic agents at tumor sites [119, 120]. In addition, the outer lipid bilayer allows further incorporation of targeting molecules, such as antibodies and aptamers capable of delivering drugs by active and/or passive targeting [118, 121, 122].

Lu *et al.* developed AS1411 aptamer-functionalized liposomes encapsulating DOX (Apt-DOX-Lip) for targeted treatment of breast cancer [123]. Enhancement of cell internalization and cyto-

toxicity afforded by Apt-DOX-Lip was observed in MCF-7 cells *in vitro* compared to controls ($P < 0.01$). In an *in vivo* model, nude mice bearing MCF-7 tumors were treated with Apt-DOX-Lip or Ctrl-DOX-Lip (25 μ g DOX equivalent per tumor via intratumoral injection) every 4 days until day 24. Apt-Dox-Lip significantly inhibited MCF-7 tumor growth compared with the Ctrl-Dox-Lip group ($P = 0.004$). Intratumoral treatment of mice with Apt-Dox-Lip caused an earlier onset of tumor inhibition on day 8 as compared with that of day 16 in Ctrl-Dox-Lip treated mice. Apt-DOX-Lip exhibited superior tumor penetration compared with the control treatment in MCF-7 xenograft models (~1000 μ m vs. ~500 μ m), contributing to the enhancement of anti-tumor efficacy. This study concluded that aptamer-functionalized drug-loaded liposomes could be a potential strategy for targeted drug delivery in anticancer treatment.

Superparamagnetic iron oxide NPs

One of the most promising and biocompatible magnetic nanoparticles is the superparamagnetic iron oxide nanoparticles (SPIONs), which possess several favourable features that are desirable for diagnostic and therapeutic applications. They can be easily degraded in humans, leading to a short body residence and lower toxicity *in vivo* [124]. The surface of SPIONs is also available for modification with polymers, metal elements and various targeting ligands for applications in nanomedicine [124, 125].

Farokhzad's group formulated a multifunctional bioconjugate composed of A10 RNA aptamer modified SPIONs and DOX (TCL-SPION-Apt) for detection and therapeutic delivery of chemotherapeutic agents to prostate cancer cells (LNCaP and PC3 cells) [126]. The cellular uptake of TCL-SPION-Apt was observed in LNCaP cells as early as 3 h after dosing followed by a time-dependent increase, while there was no obvious intracellular uptake of TCL-SPION-Apt in PC3 cells that do not express PMSA, nor was with non-targeted TCL-SPIONs. The TCL-SPION-Apt bioconjugates selectively delivered DOX to their targeted cells without significant loss in cytotoxicity. In addition, Abnous's group developed an Epirubicin (Epi)-encapsulated versatile aptamer-SPION bioconjugate (Epi-Apt-SPION) for cancer imaging and therapy *in vitro* and *in vivo* [127]. Using an *in vitro* cytotoxic assay and flow cytometric analysis, the Epi-Apt-SPION complex was found to be capable of specifically and effectively delivering Epi to C26 cells (colon carcinoma cells) but not to non-targeted CHO-K1 cells (Chinese hamster ovary cells). Epi-Apt-SPION complex is able to accumulate at the tumor and display a high MRI signal one hour post-injection. Furthermore, enrichment of

Epi-Apt-SPION at the tumor resulted in more tumor volume reduction (~2-fold and ~3.3-fold, respectively) compared with that of free Epi- and saline-treated groups.

In addition, aptamers can also be conjugated with other nanostructures for targeted and high-payload cancer therapy, such as single-walled carbon nanotubes, bovine serum albumin-based NPs, dendrimers and mesoporous silica nanoparticles [128-132] (Table 3). However, more studies on potential toxicity and *in vivo* fate of the nano-carriers are needed before successful translation of aptamer-nano-drugs from bench to bedside.

Aptamer-mediated nucleic acid therapy for cancer

Aptamer-guided nucleic acid therapy

In contrast to antibodies that can be easily inactivated due to the increased acidity in cellular organelles, aptamers generated with chemical modifications maintain stability after endocytosis [85, 88, 89, 91, 93, 145]. Aptamers have been exploited as guiding moieties for both drug delivery and nucleic acid transport vehicles such as small interfering RNA (siRNA), micro RNA (miRNA) and short hairpin RNA (shRNA) [61, 146-148] (Fig. 5). Aptamers can mediate nucleic acid therapy by increasing nucleic acid targeting efficacy for oncogenes and suppressing overexpressed signalling molecules or regulatory nucleic acids in cancer [112, 149, 150]. Intense effort has been devoted to explore aptamers as an effective nucleic acid therapy modality for cancer therapeutics.

Aptamer-siRNA chimera for targeted cancer nucleic acid therapy

Small interfering RNA (siRNA) can be integrated into RNA-induced silencing complex (RISC), contributing to the degradation of target mRNA by RNA interference (RNAi) [151]. Despite the potential of efficiently inhibiting nucleic acid expression of unlimited targets, the insufficient dose of siRNA delivered to target tissues *in vivo* hampers the translation of this strategy from bench to bedside. Generating a chimera by linking siRNA with an aptamer, aptamer-guided siRNA delivery via receptor-mediated endocytosis serves as a promising approach for cancer-targeted RNAi therapy. After transportation to the cytoplasm by a yet to be fully elucidated route or mechanism, the therapeutic siRNA can be recognized and processed by Dicer to produce the mature siRNA followed by the target mRNA degradation [152].

In order to clarify the importance of memory T cells in mediating protective immunity against cancer, Berezhnoy *et al.* used a siRNA to down-regulate intracellular mediators of CD8⁺ T cell effector differentiation [153]. To reduce the undesirable immunosuppressive effects that could result from the downregulation of siRNA in unactivated cells, they conjugated the siRNA targeting the mTORC1 component raptor to an oligonucleotide aptamer specific for 4-1BB ($K_d = \sim 40$ nM), a co-stimulatory molecule that is expressed on CD8⁺ T cells. To build up a B16/F10 melanoma tumor models, C57Bl/6 mice were firstly immunized with 10⁶ irradiated B16 melanoma cells (GVAX), and one day later mice were treated with ap-

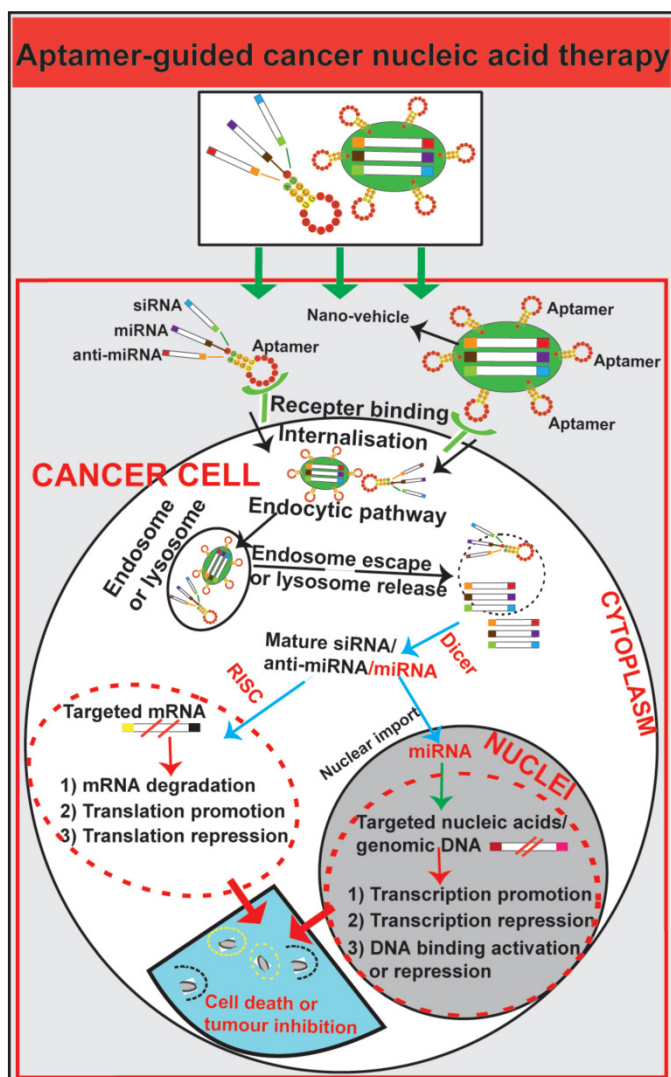


Figure 5. Schematic illustration of aptamer-guided cancer nucleic acid therapy. Exogenous therapeutic small RNAs (siRNA, miRNA and anti-miRNA) can be directly conjugated with aptamers or encapsulated within nanoparticles functionalized with an aptamer. As targeted-delivery modalities, aptamers bind to cell surface targets on cancer cells, followed by internalisation via receptor-mediated endocytosis. Through unknown mechanisms, small RNAs escape endosomes and engage cytoplasmic RNAi/miRNA protein machinery. The mature siRNA/shRNA/miRNA interact with their cytoplasmic or nuclear target sequences, leading to mRNA degradation, translation promotion or repression or modulation of gene expression.

tamer-siRNA conjugates (0.25 nmol/mouse) administered i.v. or with 1.5 µg of rapamycin injected i.p. three times daily. On day 50, the mice were xenotransplanted s.c. with 10⁶ B16/F10 tumor cells. In this study, systemic administration of the 4-1BB aptamer-raptor siRNA to mice downregulated mTOR complex 1 (mTORC1) activity in CD8⁺ T cells, leading to the enhancement of antigen-activated CD8⁺ T cell function and boosted vaccine-induced protective immunity in tumor-bearing mice. Mice treated only with rapamycin, but not with 4-1BB aptamer-conjugated siRNA, failed to reject a subsequent tumor challenge. This study indicated that aptamer-targeted siRNA downregulation of mTORC1 in CD8⁺ T cells can enhance their differentiation into memory T cells and promote antitumor immunity more effectively than pharmacologic inhibitors like rapamycin. In addition, Lai *et al.* utilised two nucleolin aptamer-siRNA chimeras (aptNCL-SLUGsiR and aptNCL-NRP1siR) to evaluate the synergistic effect in blocking key signalling pathways involved in tumor invasion and angiogenesis [154]. After establishment of an *in vivo* lung cancer model in NOD-SCID mice by subcutaneously inoculating 2 × 10⁶ CL1-5 cells, Lai and co-workers administered single chimera (2 mM of aptNCL-ControlsiR, aptNCL-SLUGsiR, or aptNCL-NRP1siR) and combined chimera (1 mM of each chimera) (aptNCL-SLUGsiR and aptNCLNRP1siR) in 50 µL PBS intratumorally three times per week until day 42. The tumor growth rate in the chimera-treated groups (aptNCL-SLUGsiR, aptNCL-NRP1siR, and combined treatment) was found to be 3-fold (with single chimera treatment) to 4-fold (with combined chimera treatment) lower than the group treated with saline. Furthermore, the combined-chimera treatment synergistically suppressed tumor invasion (1.2-fold

suppression vs. control chimera) even at half-dose administration. These data indicate that aptamer-siRNA chimera holds promise for targeted cancer nucleic acid therapy.

Aptamer-miRNA active targeting system

MicroRNA (miRNA), a small non-coding RNA with 20-22 nucleotides, regulates transcriptional and post-transcriptional nucleic acid expression and modulates normal cell functions such as proliferation, apoptosis and tumor destruction [155, 156]. The manipulation of miRNA function constitutes a promising approach for anti-cancer therapy [157, 158]. However, interaction with target nucleic acids requires the specific delivery of miRNAs to their targets to minimize side effects [157]. After conjugation of a miRNA with an aptamer, the miRNA can be specifically delivered to their targets expressing proteins recognized by the aptamer. Following internalisation into cancer cells via ATP-dependent endocytosis, miRNA is processed by Dicer followed by the loading of such miRNA duplexes into an Argonaute protein in the miRNA-induced silencing complex. Esposito *et al.* engineered a multifunctional aptamer-miRNA conjugate by linking a tumor suppressor let-7g miRNA to the GL21.T aptamer specifically recognising the oncogenic receptor tyrosine kinase Axl [159]. The results indicated that the functional let-7g miRNA was delivered by this conjugate to Axl-expressing A549 lung cancer cells and let-7g target nucleic acids could be effectively silenced both *in vitro* and *in vivo* (1.6 nmol/mouse, three injections a week for 3 weeks), contributing to the prevention of tumor cell proliferation and migration *in vitro* and the tumor growth inhibition in a lung adenocarcinoma model.

Table 3. Application of aptamer-functionalised drug-encapsulated NPs as cancer-targeted therapeutics

Aptamer	Target	Payload	Nanoparticle / carrier	Cancer	Research Group
RNA aptamer	PSMA	Dtxl, DOX, miRNA, CUR, GEM, TMPyP4, PTX	PLA-PEG-COOH NPs, SPION, Atelocollagen Liposomes, PLGA NPs, MSNs, Carbon nanotubes, AuNPs, PLGA-PEG NPs	Prostate cancer, Breast cancer, Liver cancer, Murine cancer, C6 glioma	Farokhzad <i>et al.</i> 2004, 2006, 2008, 2012 [112, 113, 126, 133] Wang <i>et al.</i> 2014 [134] Lu <i>et al.</i> 2013 [123] Kumar <i>et al.</i> 2012, 2013 [135-137] Cai <i>et al.</i> 2014 [138] Zhang <i>et al.</i> 2014 [139] Qu <i>et al.</i> 2012 [131, 140] Huang <i>et al.</i> 2014 [141] Chen <i>et al.</i> 2011 [116]
DNA aptamer	MUC1	PTX, Epi, DOX	PLGA NPs, SPION, MSNs	Breast cancer, Colon cancer	Yang <i>et al.</i> 2011 [142] Abnous <i>et al.</i> 2013 [127] Santos-Oliveira <i>et al.</i> 2013 [132]
DNA aptamer	CCRF-CEM	DOX	Nanoassembly system, DNA nanotrains	T-cell ALL	Tan <i>et al.</i> 2013 [143, 144]

ALL: acute lymphoblastic leukemia; AuMP, mesoporous silica-encapsulated Au nanorod; CUR, curcumin; Dtxl, docetaxel; DOX, doxorubicin; EGFR, epidermal growth factor receptor; Epi, Epirubicin; GEM, gemcitabine; MSN, Mesoporous silica nanoparticle; PSMA, prostate-specific membrane antigen; PTX, paclitaxel; SPION, super paramagnetic iron oxide nanoparticle.

Aptamer-mediated shRNA delivery for cancer therapy

As an alternative approach to RNAi, shRNA can be produced by various DNA constructs such as DNA plasmid, linear template, or packaged viral vectors [160, 161]. Being produced in host cells, shRNA elicits a more durable gene silencing compared to siRNA that are normally degraded around 48 h *in vivo* [162]. However, the requirement of using gene transfection or viral vectors hinders the shRNA application in clinic. To explore vector-free RNAi using shRNA, Ni *et al.* explored a chimera linking RNA A10-3 aptamer (56 nucleotides) targeting PSMA to a shRNA (50 nucleotides) against DNA-activated protein kinase catalytic polypeptide (DNAPK), a target protein for radiosensitisation [148]. After incubating LNCaP cells with aptamer-shRNA chimera (400 nM) for 2 days, the chimera significantly reduced levels of BRCA2, DNAPK and ATM mRNA. The *in vivo* efficacy of such treatment was evaluated in a xenograft LNCaP mouse model in which the mice were treated with radiation plus two intratumoral injections of 200 pmol A10-3 aptamer-shRNA. The time for reaching quadruple tumor volume was dramatically extended from one week (as in mice treated with control) to ~10 weeks. This aptamer-guided delivery of shRNA resulted in an effective knockdown of DNAPK and significantly improved tumor response to ionizing radiation.

Aptamer-mediated co-delivery system

Compared to a single agent delivery system, co-delivery of more therapeutic agents, such as chemotherapeutic agents and small RNAs, represents a more powerful approach to cancer treatment [163-166] (Fig. 6). Such a combinatorial treatment strategy may 1) provide synergistic or additive effects beyond one single drug therapy; 2) circumvent multiple drug resistance of cancer cells by targeted delivery of anticancer agents intracellularly; and 3) afford lower doses of individual drugs thus minimising systematic side effects of individual agents. A physical conjugate of DOX and A10 RNA aptamer was bridged to Dtxl-encapsulated PLGA-PEG NPs to generate a targeted co-delivery of both hydrophobic and hydrophilic drugs to tumors [106]. Both Dtxl and DOX were selectively delivered by LNCaP cells with high PSMA expression while limited DOX was internalised to the control PSMA-negative PC3 cells. In addition, anti-proliferation assays indicated that relative cell viability of LNCaP cells treated by NP (Dtxl)-Apt (DOX) was 54%, compared with that of 58%, 86%, and 100%, respectively, for cells treated with NP (Dtxl)-Apt, NP-Apt (DOX), and NP-Apt. These results suggest that the co-delivery of DOX-loaded

aptamer with Dtxl-encapsulated polymeric NPs exhibit more efficient killing of target cancer cells compared with the same amount of individual drugs.

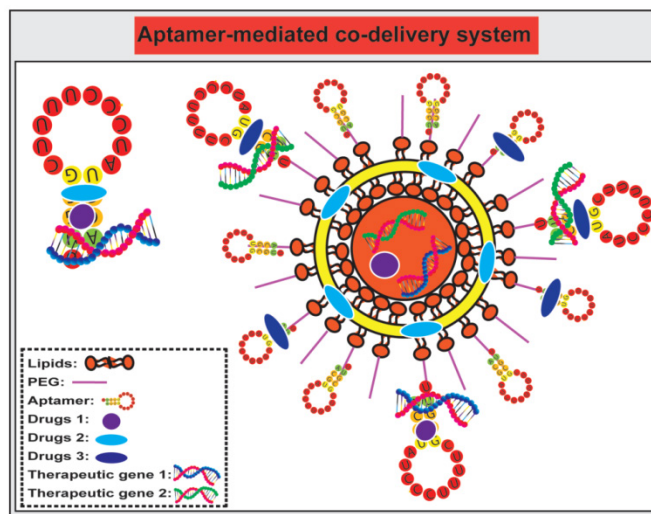


Figure 6. Schematic illustration of co-delivery of therapeutic drugs and therapeutic small RNA using free aptamer or aptamer-conjugated nanoparticles. The choice of drugs and small RNA is based on rational design and subject to extensive *in vivo* testing. Therapeutic agents can be directly conjugated with aptamers without further encapsulated into a nanoparticle, while aptamers can either be grafted on to the surface of nanoparticles as a targeting agent, and/or conjugated with a drug.

The combination of chemotherapeutic agents with small RNAs, i.e., siRNA, miRNA, anti-miRNA and shRNAs, has emerged as a potential strategy for achieving synergistic efficacies for targeted cancer therapy [167]. For example, a co-drug delivery system that combines PTX and the chimera comprising of MUC1 aptamer and let-7i miRNA specifically delivered let-7i miRNA and PTX to target ovarian cancer cells for circumventing PTX-induced chemoresistance [168]. Furthermore, Kim and colleagues synthesized PSMA aptamer-conjugated polyplexes (APs) that selectively targeted co-delivered shRNA against the anti-apoptotic gene Bcl-xL and DOX to prostate cancer cells by aptamer-mediated interaction [169]. This system efficiently activated intrinsic apoptosis of cancer cells and resulted in a 17-fold improvement in IC₅₀ compared with the simple mixture of shRNA/Lipofectamine with DOX. These results suggest that aptamer-mediated co-delivery of anticancer drugs with shRNA could broaden the therapeutic window for selective eradication of cancer cells.

Current progress in aptamer-based diagnostics

Clinical diagnostic imaging needs to use specific probes, except for nonspecific contrast agents, to interrogate a biologic target. These specific imaging probes either target a physiologic process or cellular

surface biomarkers in which the biologic targeting ligand requires high affinity, sensitivity, and specificity [170]. Aptamers possess several key advantages over proteins. The smaller size enables aptamers to achieve the enhanced tumor-to-blood and/or tumor-to-normal tissue ratio due to robust penetration into the core of the tumor, maintaining a significantly high concentration at the tumor for a clinically meaningful period and effective systemic clearance via renal filtration. Such superior sig-

nal-to-background ratio makes aptamers better imaging ligands than antibodies for effective clinical cancer diagnosis and tracking [91, 170-174]. Aptamers conjugated with diagnostic agents such as fluorescent molecules, magnetic resonance imaging (MRI) contrast enhancing agents and radioisotopes for positron emission tomography (PET) and single-photon emission computed tomography (SPECT) have been actively exploited for detecting both primary tumor and metastases (Fig. 7) [170, 175].

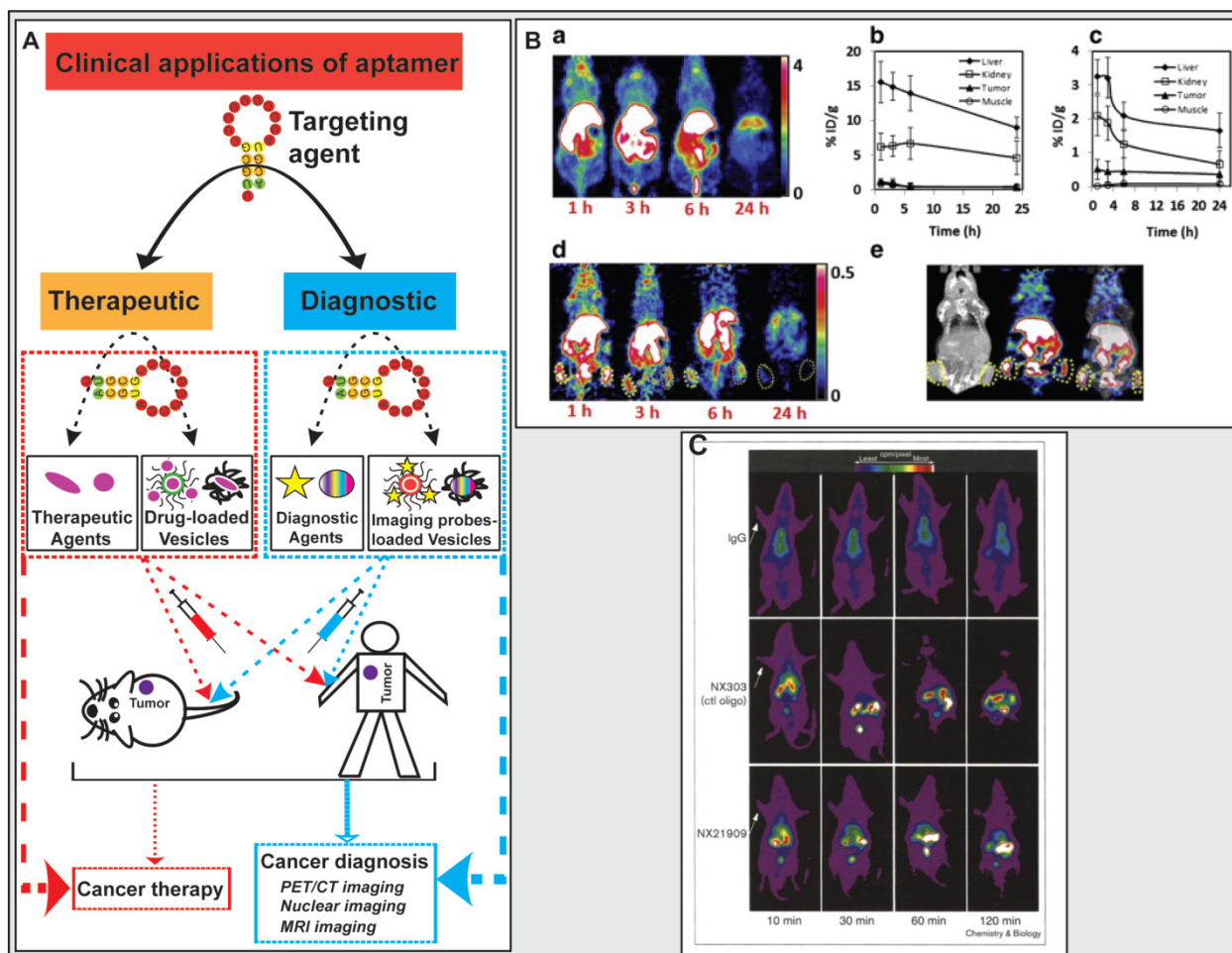


Figure 7. Application of aptamers in cancer diagnostics and therapeutics. (A) Schematic illustration of aptamer-based cancer diagnostic and therapeutic applications. (B) Representative results from the study carried out by Li *et al.* where microPET/CT images of ^{64}Cu -DOTA-AS1411 or ^{64}Cu -CB-TE2A-AS1411 at multiple time points shown that ^{64}Cu -CB-TE2A-AS1411 was stable in tumor site and was cleared rapidly from the blood, liver, and kidneys 1 h post-injection, resulting in a much higher tumor-to-liver ratio (0.56) and the tumor-to-kidney ratio (1.57) than that of 0.12 and 0.21 for that for ^{64}Cu -DOTA-AS1411, respectively. a) microPET image slices obtained at different time points for ^{64}Cu -DOTA-AS1411; b) and c) Quantitative results from PET imaging for the liver, kidney, tumor, and muscle uptake of ^{64}Cu -DOTA-AS1411 and ^{64}Cu -CB-TE2A-AS1411; d) microPET image slices obtained at different time points for ^{64}Cu -CB-TE2A-AS1411; e) Overview of microCT (left), microPET (middle), and microPET/CT fusion images (right) 1 h post-injection of ^{64}Cu -CB-TE2A-AS1411. Reproduced from Ref. [180], Copyright (2014), with permission from Elsevier. (C) Representative results from the study carried out by Charlton and co-workers where *in vivo* imaging of inflammation sites in a rat reverse passive Arthus reaction model by ($^{99\text{m}}\text{Tc}$) aptamer NX21909 indicated that the aptamer achieved a significantly higher target-to-background ratio (4.3 ± 0.6 in 2 hours) in less time than IgG (3.1 ± 0.1 at 3 hours). Reproduced from Ref. [181], Copyright (1997), with permission from Elsevier.

PET/CT imaging

Aptamer-based molecular probes have been used in PET/CT imaging. ^{64}Cu represents an attractive radiotracer that has broadly applied for *in vivo* PET imaging due to its decay characteristics and easy attachment to ligands with high affinity [176, 177]. A

chelator CB-TE2A, 4,11-bis(carboxymethyl)-1,4,8,11-tetraazabicyclo[6.6.2] hexadecane, has been known to display good kinetic stability, enhanced accumulation in tumor and reduced liver or kidney uptake, which would be a potentially effective chelator for aptamers to conjugate radiotracers for *in vivo* imaging [178, 179].

During their study of novel aptamer-mediated imaging agents for early detection of lung cancer, Li and colleagues labelled AS1411 with ^{64}Cu using various chelators (DOTA and CB-TE2A), and assessed the *in vivo* uptake characteristics of the modified aptamer for microPET/CT imaging [180]. Using a mouse H460 tumor xenograft model, 7.4 MBq (200 μCi) of ^{64}Cu -DOTA-AS1411 or ^{64}Cu -CB-TE2A-AS1411 was administrated to mice via tail vein injection followed by microPET/CT imaging at multiple time points (Fig. 7). The microCT image confirmed the presence of tumors. After the injection of ^{64}Cu -CB-TE2A-AS1411, two tumors were clearly detected by microPET imaging, with the highest tumor-to-background contrast reached at 1 h. In contrast, two tumors in mice injected with ^{64}Cu -DOTA-AS1411 were not discernable due to the high liver uptake and slow blood clearance, which were confirmed by the time-activity curves of tumor to organ ratios (liver, kidney and muscle). ^{64}Cu -CB-TE2A-AS1411 was stable in the tumor site but was cleared rapidly from blood, liver, and kidney 1 h post injection, resulting in a much higher tumor-to-liver ratio (0.56) and tumor-to-kidney ratio (1.57) than that of 0.12 and 0.21 for that for ^{64}Cu -DOTA-AS1411, respectively (Fig. 7).

Single-photon emission computerized tomography (SPECT) imaging

One earlier successful aptamer-based imaging system was to use a radiolabeled (Technetium, $^{99\text{m}}\text{Tc}$) aptamer NX21909 specifically targeting human neutrophil elastase in identifying inflammation sites in a rat model of reverse passive Arthus reaction [181]. After systemic administration, the aptamer achieved a significantly higher target-to-background ratio of 4.3 ± 0.6 in 2 hours than that of IgG, 3.1 ± 0.1 in 3 hours. The difference in tumor-over-background values is due to the rapid clearance of the aptamer signal from the blood than the IgG probe. These results provide a good example of great potential of aptamers in diagnostic imaging.

MRI imaging

In addition to their potential as excellent radio-tracers for nuclear medicine, aptamers also achieve a higher target-to-background ratio in a shorter period of time when conjugated with agents used in other imaging modalities, e.g. gadolinium-containing compounds and superparamagnetic iron oxide nanoparticles (SPIONs) [180, 182]. This is illustrated by aptamer-based contrast agents targeting vascular endothelial growth factor (VEGF) in magnetic resonance imaging (MRI) [182]. Due to its role in development of tumor angiogenesis and metastasis, VEGF165 is an ideal target for the generation of a

tumor imaging probe [183]. Radioisotope-labelled antibodies as sensitive imaging agents, such as ^{124}I -HuMV833 (humanized anti-VEGF-A mouse monoclonal antibody) and ^{89}Zr -bevacizumab (VEGF recombinant humanized monoclonal antibody), have been used for PET imaging-based assessment of cancer treatment outcomes [184, 185]. However, the *in vivo* imaging application of antibodies has been limited by their large size, immunogenicity and relatively poor tumor penetration [186]. To address these limitations, You *et al.* developed a tumor targeted MRI probe by chemically crosslinking ultrasmall superparamagnetic iron oxide (USPIO) nanoparticles (~20 nm) with an aptamer specifically targeting VEGF165 (VEGF165-aptamer-USPIO). By examining T_2 -weighted imaging ($T_2\text{WI}$) contrast enhancement, they verified the specific enhancement of MRI signal by VEGF165-aptamer-USPIO in mice bearing liver cancer xenografts [182]. Three hours after intravenous administration of VEGF165-aptamer-USPIO probe to mice, the signal-to-noise ratio of the tumor was statistically lower than that of pre-injection of the agent ($P < 0.05$), this negative enhancement phenomenon disappeared 6 h after the probe injection (Fig. 7C). In contrast, the signal-to-noise ratio of the tumor xenografts remained similar before and after the same amount of control USPIO was administrated. The results suggest that the VEGF165-aptamer-mediated USPIO probe can accumulate in the tumor, demonstrating promise for future *in vivo* diagnostic applications.

Aptamer-Guided Theranostic Nanomedicine

Theranostic refers to a strategy that integrates disease diagnosis and therapy within a single system. It is an interdisciplinary field involving chemistry, materials science, nanotechnology, molecular cell biology, pharmaceutical science, molecular imaging and medicine [187, 188]. The application of theranostics in oncology is anticipated to facilitate the selection of a safe and efficacious dose, recognition of toxic effects at the early stages of therapy, objective response monitoring and follow-up therapy planning [187, 189]. The simple form of theranostics consists of a single entity system in which a therapeutic agent is labelled with a radioisotope as exemplified by radio-labelled therapeutic monoclonal antibody, e.g. application of ^{111}In -evacizumab and ^{124}I -girentuximab in whole body biodistribution studies to quantify target accessibility [190]. However, recent work focus on a more sophisticated multi-component system in which targeting, imaging, diagnostic, and therapeutic functions are assembled in smartly engineered nanocarrier. Such theranostic nanoparticles capitalise

on the advantages of nanoparticles, including high surface area, high payload capacity, prolonged circulation half-life and increased delivery to tumors [191, 192]. The commonly used tumor targeting agents are antibodies. However, the large size and other limitations associated with antibodies demand the development of alternative targeting moiety for theranostics. Aptamer-functionalised nanoparticles emerge as promising targeting agents for theranostic applications due to the sensitive tracking and imaging of aptamers *in vivo* and the ability to facilitate drug transport across physiological barriers [62, 193, 194].

The innovative approaches to aptamer-guided theranostics are exemplified by an Au@Ag/Au nanoparticles assembled with activatable aptamer probes (AAPs) developed by Jia and co-workers [193]. The therapeutic arm of the system consists of a photothermal therapy (PTT) entity composed of Au@Ag/Au nanoparticles capable of large absorption in the near-infrared (NIR) window followed by efficacious hyperthermia; while the diagnostic arm of the system is made of AAPs that target human A549 lung cancer cells. The fluorescence of the labeled activatable aptamer is quenched in sites where there are no tumors by a fluorescence-labeled DNA (F-cDNA) co-grafted onto the same nanoparticles. Once the aptamer-Au@Ag/Au nanoparticle binds to tumor cells and interacts with the target protein on A549 cells, the quencher fluorophore on the suppressor F-cDNA moves away from the aptamer-Au@Ag/Au nanoparticle, leading to the activation of the fluorescence from

the A549-targeting aptamer. The subsequent tumor-specific fluorescence imaging allows the precise positioning of the NIR light towards the tumor to facilitate *in vivo* guided photothermal therapy (Fig. 8B). The authors demonstrated the utility of this novel theranostic therapy in an A549 mouse xenograft model. The tumor bearing mice were administered i.v. with 300 μ L of the aptamer-nanoparticles (1.1×10^{10} particles per mL). Mice without tumor or non-targeted tumor-bearing mice had no or minimal activated fluorescence from the aptamer, while prominent fluorescence signals accumulated rapidly in A549 tumor sites 5 min after administration and reached to the peak at around 15 min (Fig. 8C). The relative highest-contrast tumor imaging signals appeared at around 105 min. The subsequent efficacy of inducing tumor necrosis was achieved after exposing the tumor to a 980 nm laser light. Prominent necrosis was observed in A549 tumors treated with irradiation, and the tumor growth was inhibited at necrotic sites (Fig. 8C). In light of the broad application of Au@Ag/Au NPs and the short development circle for aptamers, aptamer-mediated imaging-guided PTT strategy might be explored to a flexible platform for real-time detection and treatment of a wide range of solid cancers. The incorporation of chemotherapy agents into this system will further facilitate the transformation of this platform to a synergic anti-cancer strategy by combination of aptamer-guided chemotherapy and PTT.

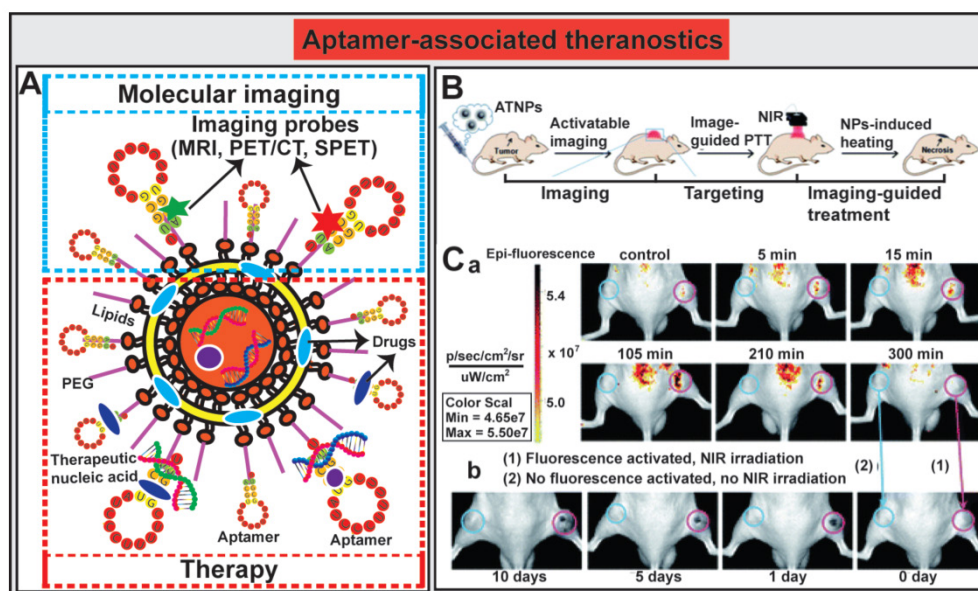


Figure 8. Aptamer-guided theranostic nanomedicine. (A) Schematic representation of aptamer-associated theranostic nanomedicine. (B) Schematic illustration of the novel activatable theranostic nanoprobe (ATNP) strategy for aptamer-mediated imaging-guided photothermal therapy (PTT) *in vivo*. (C) *In vivo* aptamer-related activatable fluorescence imaging (a) and imaging-guided site-specific NIR PTT (b) of SPCA1 (cyan circles) and A549 (pink circles) tumor following ATNP administration. Reproduced from Ref. [193]. Copyright (2014), with permission from The Royal Society of Chemistry.

Future perspectives

Tumor heterogeneity: another obstacle for anticancer treatment

In addition to killing cancer cells, current chemotherapy or radiotherapy can also damage normal cells. Such intensive therapies can also alter the cancer cell ecosystem in which variant or drug-resistant cancer cells emerge. The altered cancer ecosystem can be explained by the evolving cancer models. The conventional stochastic cancer model suggests that each cell within a tumor has equal potential to propagate and maintain a tumor but it is hard to prospectively identify the tumorigenic subset [195-197]. Recent studies suggest that cancer can be considered as a hierarchical organisation [198], which is the basis of the cancer stem cell (CSC) model. In the CSC model, only a small population of cancer cells possesses the ability to generate a tumor. Generally, CSCs, as 'roots of cancer', are defined by their abilities to form new tumors that histologically resembles the original tumor when xenotransplanted into immunodeficient mice. However, their descendant cells lose their self-renewal potential through either asymmetric or symmetric division and proliferate extensively [199-202]. The CSC model provides a plausible account for poorly understood clinical phenomena, such as drug resistance to chemical- and/or radiotherapy, as CSCs can resume growth and lead to a new relapse after primary therapy [198]. However, a new concept - the dynamic CSC model - has emerged to indicate that the CSC phenotype is much more fluid than previously predicted and can be regulated by external signals [203]. It suggests that not only can CSCs self-renewal create to differentiated cells, but the de-differentiation of non-CSCs to CSCs can also occur and thus revert to CSC [1, 204, 205]. The observation of inter-conversion between CSCs and their offspring suggests the necessity to evolve current anticancer strategies to target both types of cancer cells with self-renewal potential and the bulk differentiated bulk cancer cells [206].

Aptamer-mediated chemotherapy for eradicating CSCs

Based on current studies, CSCs are attractive targets for anticancer therapies. Limitations, such as only a small population of CSC, overlapping of CSC-correlated surface markers and signal transduction circuits (e.g. Wnt and Notch pathway) with normal stem cells, hamper the effective targeting of CSCs [207]. However, cell surface markers and/or signal transduction routes that underline tumor clonogenicity and tumor resistance could still provide promising therapeutic targets for developing effective

anticancer drugs [208]. One of the challenges facing effective anti-CSC therapy is the elevated expression of drug efflux pumps and intracellular detoxification capacities [206, 209-214]. Active targeting of CSCs might hold the answer for circumventing chemoresistance [215]. Emerging data indicate that sensitizing CSCs to current chemotherapy using a CSC-targeting strategy can eliminate CSCs and minimize cancer recurrence [216]. For example, McAuliffe *et al.* have shown that targeting CSC-associated Notch pathway with a γ -secretase inhibitor and Notch3 siRNA knockdown can increase tumor sensitivity to platinum treatment in both cancer cell lines and patient-derived xenograft ovarian cancer models [217]. In addition, following the isolation of CSCs from non-small-cell lung carcinoma (NSCLC) sphere cells and blocking the stem cell factor (SCF) c-kit signalling that modulates self-renewal and proliferation of lung CSCs, the anti-SCF antibody conjugated cisplatin could target both non-CSCs and CSCs, enhancing the anti-tumor efficacy in human NSCLC [218]. Instead of developing new drugs, these studies explored a potential effective strategy that utilises a CSC-targeting moiety to deliver existing chemotherapeutic agents to eradicate both non-CSCs and CSCs. Most recently, RNA aptamers for targeting CSC markers (EpCAM and CD133) have been isolated [49, 60, 219]. Upon binding to cancer cells expressing these CSC markers, these CSC-targeting aptamers efficiently internalised via receptor-mediated endocytosis, an important mechanism capable of bypassing ATP-binding cassette transporters (ABC transporters) that are responsible for multiple drug resistance in CSC [60, 100]. Indeed, by conjugating a cytotoxic agent to CSC-targeting RNA aptamers, we have been able to transform a classic chemotherapy agent into an effective CSC killer, circumventing drug resistance in CSCs and eradicating both non-CSC and CSC *in vitro* and *in vivo* (our unpublished data).

Conclusions

Aptamers as chemical antibodies possess attractive physicochemical and cell biological attributes for clinical applications both in cancer diagnosis and targeted cancer therapy. In particular, aptamers have been exploited as active targeting moieties for targeted delivery of therapeutic agents for cancer therapy. Through either direct physical conjugation or chemical conjugation onto nanoparticles, aptamers can guide drugs to tumor sites efficiently with superior tumor penetration than antibodies. The co-delivery of classic anticancer reagents coupled with single or multiple small RNA therapeutics may offer more efficacious cancer treatment regimens than either of these anticancer tactics alone. Further engi-

neering of aptamers with shorter sequences but extended blood residence time and superior tumor penetration will facilitate the development of aptamer-guided cancer therapeutics with significantly improved clinical outcome and better quality of life for patients with cancer.

Abbreviations

2'-OMe, 2'-O-methoxy; ABC transporter, ATP-binding cassette transporter; AG, arabinogalactan; Apt, aptamer; AAP, activatable aptamer probe; CEA, carcinoembryonic antigen; Control, Ctrl; CSC, cancer stem cell; CT, computed tomography; DNAPK, DNA-activated protein kinase catalytic polypeptide; DOX, doxorubicin; Dtxl, docetaxel; EGFR, epidermal growth factor receptor; EpCAM, epithelial cell adhesion molecule; Epi, Epirubicin; EPR, enhanced permeability and retention; F-cDNA, fluorescence-labeled DNA. IFP, interstitial fluid pressure; IL4Ra, IL-4 receptor- α ; Lip, liposome; MDSC, Myeloid-derived suppressor cells; miRNA, micro RNA; MRI, magnetic resonance imaging; MSC, mesenchymal stem cell; MSN, Mesoporous silica nanoparticle; mTORC1, mTOR complex 1; NIR, near-infrared. NP, nanoparticle; NSCLC, non-small-cell lung carcinoma; PEG, Polyethylene glycol; PET, positron emission tomography; PLGA, Poly(Lactide-co-Glycolide); PSMA, prostate-specific membrane antigen; pSTAT6, phosphoSTAT6; PTX, Paclitaxel; PTT, photothermal therapy. RISC, RNA-induced silencing complex; RNAi, RNA interference; SELEX, systematic evolution of ligands by exponential enrichment; shRNA, short hairpin RNA; siRNA, small interfering RNA; SNR, signal-to-noise ratio; SPECT, single-photon emission computed tomography; SPION, superparamagnetic iron oxide nanoparticle; T2WI, T2-weighted imaging; TAM, tumor-associated macrophage; USPIO, ultrasmall superparamagnetic iron oxide; VEGF, vascular endothelial growth factor.

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Competing Interests

The authors have declared that no competing interest exists.

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