Use of Aptamers for Targeted Theranostic in Cancer

Subjects: Biotechnology & Applied Microbiology

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Aptamers are short, single-stranded oligonucleotides synthesized in vitro from a randomized oligonucleotide library against a specific target. These molecules are capable of binding to a wide range of biological targets with high specificity and affinity. They present great advantages over antibodies with potential applications in research, diagnosis, and therapeutics. Specifically for tumors with late-stage identification and poor prognosis, like pancreatic cancer, the study of novel aptamers holds tremendous potential for cancer diagnosis and treatment. Along with cancer treatment, aptamers have also shown high potential in regulating the immune response and modulating several critical steps of signaling cascades, such as in immune checkpoints.

aptamer cancer

1. Introduction

Aptamers are short synthetic single-stranded ribonucleic acid (ssRNA) or desoxyribonucleic acid (ssDNA) oligonucleotides, selected through an in vitro method called Systematic Evolution of Ligands by Exponential enrichment (SELEX) [1][2]. They can display high specificity and affinity to a broad spectrum of target molecules, from ions or small molecules to proteins, viruses, bacteria/pathogens, cells, and tissues.

The SELEX process was developed in 1990, simultaneously by two independent groups, Tuerk and Gold [3] and Ellington and Szostak [4], with the goal of isolating RNA sequences that would specifically recognize target molecules. Since then, many improvements have been made in this procedure to increase its selectivity and shorten the selection process [5]. Aptamers possess a vast field of applications, including diagnosis, therapy and drug delivery systems, food safety, and environment monitoring [1][2][6].

The conventional SELEX involves subsequent cycles of incubation, binding, partitioning, and amplification (**Figure 1**). It usually starts with a synthetic pool of ssDNA or RNA oligonucleotides called the primary library, which can comprise up to 10^{15} different sequences. This initial pool is incubated with the target molecule (in a free or immobilized form), under specific conditions of incubation. Unbound or weakly bounded sequences are eluted (partitioning) from the target and the target-bounded sequences are then retrieved and amplified by PCR or RT-PCR, for DNA or RNA libraries, respectively. The process is repeated for several rounds (between four and 20), with increasingly stringent conditions, in order to obtain a final pool with low heterogeneity and high affinity of

sequences is achieved. The enriched oligonucleotides obtained are finally sequenced and their binding affinities to the target are assessed to find the best sequence [1][2].

Figure 1. SELEX usually starts with the incubation of the target molecule and a primary library composed of ssRNA/ssDNA oligonucleotides under specific conditions. Unbound sequences are eluted, and target-bounded sequences are amplified by PCR or RT-PCR. These selected sequences are used in subsequent SELEX rounds until the population achieves the required affinity. The final product is sequenced and the affinity of aptamer-target complex is characterized.

The affinity of aptamer–target complex can be characterized by label-free or label-based strategies, through the calculation of dissociation constant (Kd), conformational changes, kinetics, and thermodynamics [7].

Aptamers can exhibit high affinity and low Kd (usually in pico- to nanomolar range) [2], with a similar or even superior target binding affinity than antibodies. In fact, they are often mentioned as chemical antibodies and offer several inherent advantages, as they can be produced over a wide range of targets, display low toxicity and immunogenicity, low molecular weight, good solubility and tissue permeability, high tolerance to environmental conditions (temperature, pH), conformational flexibility, easy modification and conjugation with other molecules, as well as an economical and reproducible synthesis, avoiding animals' dependency for production. Once an aptamer is selected and characterized, it can be produced at a large scale, at low cost, and with low batch inter variability [1][2]. Therefore, numerous aptamers have been selected and applied for cancer, immune, and microbiota research.

2. Cancer Therapeutics

Aptamers can be applied as therapeutic agents acting mostly as antagonists, binding to the target, and blocking its function, but they can also be applied as agonists, stimulating the targets' function [8][9][10]. As therapeutic agents, the features of aptamers potentiate their utilization on drug delivery systems for human disease treatment [9][11], as they are capable of transporting a variety of therapeutic agents as small molecules, peptides, or toxins [11].

However, in vivo applications of aptamers actually possess some issues. As an example, the conformation of the target during in vitro selection could be different to the conformation under in vivo conditions, affecting its ability in binding. Also, they have short half-life in vivo due to nuclease sensitivity and fast renal filtration [9][10][12][13].

Nowadays, cancer remains one of the deadliest diseases, with a limited number of reliable biomarkers, as well as reduced specificity, affecting healthy tissues. Examples such as chemotherapy or antagonism, like DNA damage repair drugs, can induce modification in the microenvironment, which can lead to an opposite effect, triggering invasion and/or tumor metastases [6].

In view with this, more efficient, specific, and refined therapies are being studied, targeting different hallmarks of the cancer. One of the most promising is the use of oligonucleotide aptamers, which can bind to specific membrane receptors to start the intended action, having already shown solid results against tumor growth and metastatic capacity, as well as modulating the tumor microenvironment to become more susceptible and easier to treat. This methodology also allows combination with other molecules, such as antibodies, which can enhance and potentiate the response. In fact, studies have shown higher biocompatibility and more refined targeting toward the tumor site, also reducing the possibility of developing resistance to treatment [6].

For breast cancer, some aptamers have been studied. HeA2-3 is an aptamer used to target HER2, one of the most relevant receptors against this cancer, which inhibits the growth and survival of breast cancer cells [14]. PNDA-3 aptamer targets periostin and effectively inhibits breast cancer cell invasion and metastasis in vivo, despite limited in vitro growth effects [15] Two aptamers target the MNK1, an essential component for cancer growth, apMNK2F, which has proven to be effective in decreasing both tumor size and the incidence of metastases in murine breast cancer models [16], and apMNK3R [17] The aptamer ex-50.T is used to target the exosomes released by cancer tissue, reducing the molecular pathways that promote cancer proliferation such as AKT/Pi3K pathway [18]. In addition, 2′-Fluoropyrimidines-RNA aptamer is used conjugated with nanoparticles and allows treatment with photothermal therapy in triple-negative breast cancer cells [19]. The AS1411 aptamer targets nucleolin, which leads to the reduction in angiogenesis capacity, i.e., the capacity of producing new vessels to create a better microenvironment for tumoral development [20].

In colorectal cancer, four aptamers with the highest potential were identified. These were aptamer YJ-1, which binds to Carcinoembryonic antigen (CEA) and death receptor 5 and leads to the inhibition of tumor growth and migration potential [21]; apPDGF-BB, which is an aptamer for PDGF-BB and is important in reducing proliferation capacity [22]; another aptamer studied is apPD-1, which targets the PD-1 receptor, which leads to the activation of immune system [23]; and the last aptamer is NOX-A12, which inactivates CXCL12, showing promising results in the following studies [24].

Lung cancer is another cancer with high concern for new therapeutics due to its aggressiveness and lack of early detection strategies. However, several candidates have been identified as promising recognition elements. For example, aptPD-L1 targets the PD-L1 receptor and regulates the tumor microenvironment, stimulating the immune system cells [25] and AP-74 M-545 aptamer is used against galectin-1, also stimulating the immune system. In LL/2 tumors in vivo, it effectively inhibits tumor growth by reducing T cell apoptosis [26]. Two aptamers known as TBA353 and TBA535 target TBA, leading to reduced proliferation and invasive potential in lung cancer cell lines [27] and a PEGylated aptamer called RA16 is used specifically to bind to non-small-cell lung cancer cells, leading to tumor growth suppression [28].

Cancer of the liver also has some potential aptamers. For example, aptamer AS1411 [29] and AS1411-modified [30] target nucleolin and galectin-14, respectively, leading to a reduction in the tumoral features, in both in vitro and in vivo models, and the CL-4RNV616 aptamer targets EGFr, reduces proliferation, and promotes cell apoptosis [31].

Considering renal cancer, some aptamers are described. SW-4 and SW-4b are two aptamers used to modulate the endocytosis process of renal cancer cells, inducing their apoptosis and cell cycle [32]. The aptamer AS1411, currently in phase II clinical trials which have shown high potentiality in other cancers, can also induce a reduction in aggression [33].

Regarding prostate cancer, several studies have focused on the finding of new aptamers, since this cancer affects millions of men. Apt63 aptamer targets APT5B to reduce its metastatic potential and leads to cancer cell death, with no toxicity in non-tumoral cells, both in cell lines and in xenograft models [34]; two aptamer target PSMA, namely A9g [35] and other PSMA-specific aptamers [36], which is considered to have high potential against this cancer type, since PSMA is specific to prostate cancer tissue; combining A9g with AGRO100, the inhibition of cancer features seems to be highly improved [37]; CG3 is an aptamer that inhibits the proliferation of blood cancer cells originated in prostate, showing potential both as a diagnostic tool for prostate cancer and as a drug delivery therapy system [38]; the AS1411 also expresses impact on prostate cancer [39]; finally, the shRNA/PEI-PEG-APT/DOX conjugates can detect and inactivate PSMA-positive cancer cells [40].

Regarding other types of cancer, there are aptamers that exhibit potential in treatment and/or diagnosis. For glioblastoma, 40L and A40s target CD133 and allow the reduction in metastasis process [41]; in oral cancer, the inhibition of Heparinase by the anti-HPSE1 seemed to be effective to reduce invasion capacity and lead to cytotoxicity of cancer cells [42]; regarding cervical cancer, A2 aptamer targets E6 and E7 oncogenes, which lead to tumor regression and growth inhibition [43]; concerning bladder cancer, alncRNA aptamer inhibits TF transcription and oncogene activity, leading to reduced cancer features [44]; and, in view of gastric cancer, the use of trimeric aptamer targets to ErbB-2/HER2, showing high efficiency when combined in the trimeric form, reducing endocytosis and growth rate [45]. Leukemia has some potential aptamers, such as AS1411 [46], apβ-arrestin2 for the reduction in the progression of chronic myeloid leukemia [47], and ssRNA MLL-1 to target MLL-1, leading to cancer growth reduction [48]. For Hodkin's lymphoma, an aptamer for CD30 is used to diagnose the disease, namely the CD-RNA aptamer with gold nanospheres called HAuNS [49]. In the myeloma disease, the use of NOX-A12, which targets CXCL12, leads to a reduction in cell adhesion and signaling processes, as well as cancer cell

growth and proliferation [17], a phase IIa study in 28 relapsed/refractory multiple myeloma patients found NOX-A12, whether given alone or with bortezomib/dexamethasone, to be safe and well-tolerated [24]. Regarding skin cancer, F3B aptamers inhibit MMP-9, which reduces cell proliferation and extracellular matrix remodeling as well as allowing of the detection, being a theragnostic aptamer [50].

Pancreatic cancer is one of the leading causes of cancer death worldwide and is often diagnosed at an advanced stage with local invasion and distant metastases, which contribute to the high mortality rates associated with the disease [51][52]. The lack of effective treatments and diagnostic methods further compounds the problem, underscoring the need to develop targeted therapeutics and diagnostic approaches to improve patient outcomes [53]. Aptamers are, therefore, an attractive option for developing more accurate diagnostic tests and more effective treatments for pancreatic cancer.

The E07 aptamer, which targets the epidermal growth factor receptor (EGFR), can be used to improve the effectiveness of gemcitabine, having the potential to be a more effective treatment for pancreatic cancer than current therapies using gemcitabine alone [54]. Two RNA aptamers (P19 and P1) were selected to specifically recognize pancreatic cancer cells and deliver a duplex C/EBPα-saRNA molecule to target tumor growth suppression. In animal models, P19-C/EBPα-saRNA induced a 40% decrease in tumor growth with no toxicity [55]. A TfR aptamer-C/EBPα-saRNA conjugate used to treat advanced PDAC in a mouse model, also showed a significant reduction in tumor size [56]. Aptamer XQ-2d enables the conjugate to specifically recognize and enter PDAC cells, minimizing its side-effects on normal tissues. XQ-2d-His-SH2 CM-(Arg)9 conjugate impairs the ability of pancreatic cancer cells to undergo epithelial-mesenchymal transition (EMT), inhibiting the proliferation, invasion, and metastasis of PDAC cells [57].

SQ2, is an RNA aptamer with the capacity to be internalized by tumoral cells through ALPPL2-mediated clathrin-independent pathways [58]. This aptamer is capable of carrying a cytotoxic drug, demonstrating potential applications in imaging and therapeutics for all cancers that have aberrant expression of ALPPL2 [58][59]. The aptamers (1 and 146) colocalized with existing Cancer Stem Cell (CSC) markers, such as CD44, CD24, EpCAM, and CD133, which can recognize CSC marker-enriched Circulating Tumor Cells (CTCs) in pancreatic cancer, suggesting potential applications in pancreatic cancer diagnosis and treatment [60]. The X-aptamer targeting the Thy-1 membrane glycoprotein (THY1), overexpressed in pancreatic cancer cells, provides a non-immunogenic and specific option in the context of biomarker development and clinical application [61].

Additionally, some other aptamers are being tested. The aptamer aptacoy binds to the TfR, leading to an enhancement in the therapeutic response of Doxorubicin [62]; NOX-E36 aptamer connects to CCL2 and can be used not only in pancreatic tumors, but also in Diabetes mellitus type 2 (T2DM) [63]; cancer cell secretome can be detected and differentiated from healthy cells using the cyclophilin B aptamer [64]; the P12FR2 aptamer binds to PAUF and reduces the growth and metastatic capacity of cancer cells [65]; the use of NOX-A12, which targets CXCL12, leads to the reduction in cell adhesion and signaling processes, as well as cancer cell growth and proliferation [53].

Aside from the specificity for cancer sites, there are some aptamers that are not specific to target tissues, but cancer cells. The aptamers 4-1BB, which targets TNFSF9 [66], and OX40, which binds to TNFSF9 [67], promote immunotherapy treatment. GL21.T is another aptamer which allows the improvement of cancer cells to endocytose drugs, as well as to promote tumor selectivity [68]; Two different aptamers, Apt-siRNA [69] and CAR-like multivalent aptamer [70], do not have a specific target, although they have the capacity to lead to anticancer effects; bi-functional aptamers target opsonin C3b/iC3b, resulting in cancer cell destruction [71]; the AS1411 aptamer and cyclic peptide RGD bind to Alpha-v beta 3 (αvβ3), inhibiting cell growth and proliferation [72]; the H02 aptamer targets α5β1, reducing invasion capacity [73]; and dox-incorporated multivalent aptamer-siRNA conjugates produce powerful anticancer effects [74].

Conversely, tumor cells express shared tumor-specific antigens, which can induce immune responses when presented with MHC molecules on the cell surface. MAGE-A3 is a tumor-specific antigen that is highly expressed in several types of cancer, including melanoma, esophageal carcinoma, gastric carcinoma, multiple myeloma, breast cancer, and pancreatic cancer, often associated with malignancy and poor prognosis. Wang et al. (2016) described a DNA aptamer against the shared tumor-specific antigen MAGE-A3, which discriminates multiple types of cancer cells from normal cells (Ap52) [75]. In 2021, Wang and his team demonstrated that Ap52 can effectively penetrate cancer cells and selectively deliver the anticancer drug Doxorubicin (Dox), causing cytotoxicity to multiple types of cancer cell, becoming a promising therapeutic delivery system for various tumors [76]. In addition, aptamers may provide combinatorial manipulation of cell immunity and drug targeting for cancer therapy, and a combination of aptamers targeting various shared tumor-specific antigens may be used to combat tumor heterogeneity [76][77].

The versatility of aptamers created an important opportunity for diverse application in the field of immunology both in diagnosis and therapy. Considering diagnostic techniques aptamers might be used for two main purposes: detection [49][78][79] and separation [80][81][82]. In therapy, aptamers have two other principal functions that include being a therapeutic drug itself [83] or being responsible for taking fewer drugs to treat the affected cell or organ and, in this way, reducing the need for higher doses of drugs that have relevant secondary effects [84][85][86].

The gold standard techniques for immune diagnosis are flow cytometry and Enzyme-linked Immunosorbent Assay (ELISA) [87] and aptamers present features useful for both strategies, offering a broad range of solutions for the detection of biomolecules like proteins, including immunoglobulins or membrane receptors [49][83][85][88], cells [80][81][89], or even Extracellular Vesicles (EVs) that have gained popularity in fighting diseases like cancer. For EVs, CD63 is being consistently used as one of the most promising targets for aptamers on the surfaces of EVs [79][82]. Aptamers might also be used for cell separation using, as an example, sorting equipment accoupled to flow cytometry, which is useful both as a diagnosis technique [80][81] and also for therapy purposes in techniques like immunotherapy or HIV therapy [80][90].

Considering therapeutical strategies, aptamers might be a drug by themselves and although there is potential for exclusive use of aptamers as a therapy [83], the most common approach is using aptamers together as co-adjuvant therapy. For example, aptamer- T cells together with anti-PD1 immune-checkpoint monoclonal antibodies have a

synergetic effect in reducing cancer, in vitro and in rats, and aptamer-linked small-interfering RNA chimeras to selectively knock down genes in mouse breast cancers [91][92]. This approach allows a decrease in dose in immunotherapy applications and, in this way, reduces the secondary effects of therapies that might cause a lot of discomfort and distress in patients [83,93,94]. Aptamers are used not only in order to destroy the disease agent, like a cancer cell [89], but are often used as immune modulators, reducing immune activity [86], targeting or influencing immune checkpoint inhibitors [89][93][94], and/or promoting immune cell activity and infiltration [91][95].

Aptamers are also showing a lot of potential for drug delivery therapies, not only in immune context, but in general. This happens because aptamers are very versatile as drug-targeting tools and can be used with at least three relevant functions. Aptamers might be carriers for other drugs, allowing the encapsulation of drugs [96], be used for targeting, allowing the drug to reach in higher quantity the compromised cell/organ [84][85][86], and also might be used as a gating system that induces the release of the therapeutical drug when arriving at the target [96].

The microbiota plays a crucial role in protecting against pathogens and preventing infections, competing with pathogenic microorganisms for resources and space and preventing them from colonizing and infecting the host, producing antimicrobial peptides that can kill or inhibit the growth of pathogenic microorganisms and modulate the immune system's response to pathogens [97][98]. However, when a disruption of this microbiota occurs, it can lead to harmful situations, particularly respiratory, gastrointestinal, and skin infections [97]. One example is antibiotics that can disrupt this balance, promoting pathogenic bacteria overgrowth and increased risk of infection, with *Streptococcus* and *Staphylococcus* being among the most common [99]. Also, the overgrowth of Clostridium difficile can lead to a severe infection, which can cause symptoms ranging from diarrhea to life-threatening complications [100].

Aptamers have been increasingly used in microbiome diagnostics, where they can identify and characterize the microorganisms present in a specific environment, such as the human gut, skin, or oral cavity, because of their unique binding properties and their ability to detect specific microbial biomarkers in complex biological samples [1] Their use in the gut microbiota field has several advantages, including high sensitivity and specificity, easy modulation and synthesis, low cost, and high stability, making them valuable tools for studying the complex interactions between the microbiota and the host and help to develop personalized microbiota-based therapies [1][2].

One application of aptamers in microbiota research is the identification and quantification of specific microbial species or strains [101]. Aptamers can be designed to specifically bind to the surface molecules or antigens of certain bacteria or other microorganisms, facilitating the identification and quantification of those organisms [101]. This method can be particularly useful in identifying and monitoring specific pathogenic or beneficial microorganisms in complex microbial communities, with studies by Hu et al. and Song et al. describing this novel approach in strains like *Bifidobacterium bifidum* (CCFM641-5) and *Lactobacillus casei*, at the same time, demonstrating the potential of aptamer-based strategies for the rapid and accurate detection and quantification of bacteria in food products [102][103].

Another application of aptamers in microbiome research is the study of microbial metabolites and signaling molecules, where aptamers can be designed to specifically recognize and bind to small molecules, such as short-chain fatty acids, neurotransmitters, and other signaling molecules produced by the microbiota, where aptamers can profile and characterize at the species, strain, or function level. They can be used in combination with high-throughput sequencing technologies, such as 16S rRNA sequencing to identify and quantify specific microbial populations in the gut microbiota and to identify and quantify specific microbial metabolites, which are important for gut health [104]. This approach allows researchers to better understand the interactions between the microbiome and the host and how they influence overall health and disease, providing a more accurate diagnosis and better understanding of the disease [105][106][107][108][109].

The use of aptamers in gut microbiota research is still in its early stages and there is significant potential for future applications. Aptamers have the potential to be used as a diagnostic tool for gut microbiota-related disease, such as inflammatory bowel disease and colorectal cancer, but also for gut microbiota-related diseases, such as antibiotic-resistant infections [109]. Moreover, aptamers can be integrated with other technologies, such as high-throughput sequencing and mass spectrometry, to provide a more comprehensive analysis of the gut microbiota [110]. This approach has the potential to provide a more detailed understanding of the gut microbiota and its role in human health and disease.

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