Aptamers and Cancer

Terrence P McGarty
White Paper No 150
May, 2018

Aptamers are short oligonucleotides which can be selected to adhere to specific cell targets. We examine herein their progress and their application to cancer treatment. We have a focus on prostate cancer.

Notice

This document represents the personal opinion of the author and is not meant to be in any way the offering of medical advice or otherwise. It represents solely an analysis by the author of certain data which is generally available. The author furthermore makes no representations that the data available in the referenced papers is free from error. The Author also does not represent in any manner or fashion that the documents and information contained herein can be used other than for expressing the opinions of the Author. Any use made and actions resulting directly or otherwise from any of the documents, information, analyses, or data or otherwise is the sole responsibility of the user and The Author expressly takes no liability for any direct or indirect losses, harm, damage or otherwise resulting from the use or reliance upon any of the Author's opinions as herein expressed. There is no representation by The Author, express or otherwise, that the materials contained herein are investment advice, business advice, legal advice, medical advice or in any way should be relied upon by anyone for any purpose. The Author does not provide any financial, investment, medical, legal or similar advice in this document or in its publications on any related Internet sites.

Furthermore, this document contains references to and quotes from papers and documents under the premise of Fair Use in order to present ideas and understandings in context. The Author has attempted to make any and all references to such material separate from those of the author per se and has referenced the source expressly in all cases. These documents are for the dissemination of ideas and have no commercial intent. The Author would appreciate any communications relating to these documents and these should be sent to:
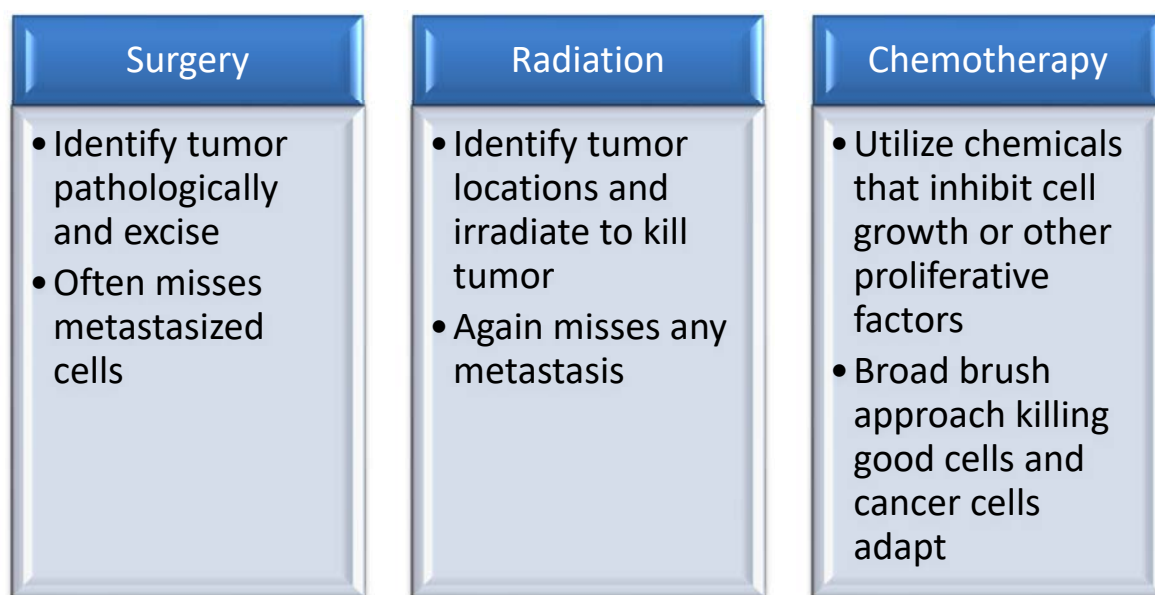
tmcgarty@telmarc.com.

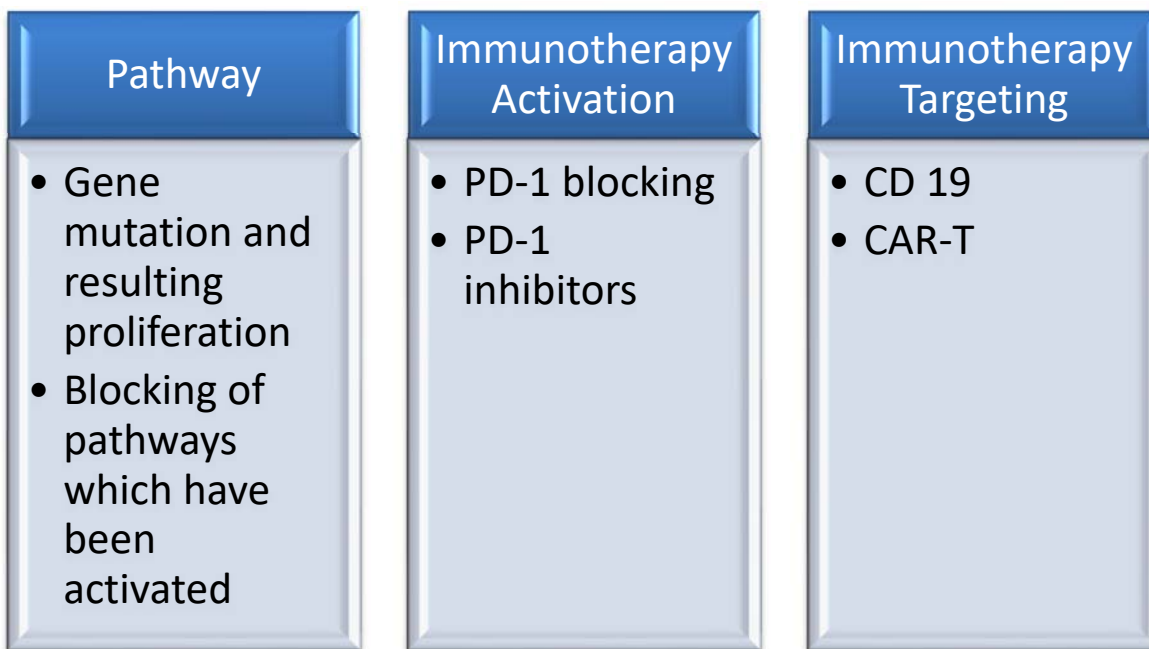# Contents

1   INTRODUCTION

Current cancer therapy consists of a multiplicity of new tools allows improved specificity on targeted cells. Dealing with cancers has been a slow but steady process, from not having it well defined, to a period of assuming that all cancers were alike, to the current state of a modest genetic understanding of cancers. The tools are now available, and expanding rapidly, that allow for a better understanding of cancer's development and expansion, and to ascertain how best to mitigate the uncontrolled growth.

The classic methods were broad brush techniques, using what was available in the then existing tools. We show some examples below:

Surgery	Radiation	Chemotherapy
• Identify tumor pathologically and excise • Often misses metastasized cells	• Identify tumor locations and irradiate to kill tumor • Again misses any metastasis	• Utilize chemicals that inhibit cell growth or other proliferative factors • Broad brush approach killing good cells and cancer cells adapt

Classically it was assumed that the lesion, tumor, growth could be excised and that if one had done a good job then this would eliminate the cancer. Sometimes it worked, yet more often the cancer reoccurred. The question was thus; what was the basis for metastasis and how quickly does it start after the initial lesion.

In contrast the new therapies respond to the specifics as seen in the cancer cells. For example in CML the breakdown of the kinase path, in melanoma the BRAF mutation, or using immunological techniques in blocking PD-1 inhibition or in targeting CD-19 in hematological cancers with CAR-T cells. We demonstrate these techniques below:

Pathway	Immunotherapy Activation	Immunotherapy Targeting
• Gene mutation and resulting proliferation • Blocking of pathways which have been activated	• PD-1 blocking • PD-1 inhibitors	• CD 19 • CAR-T

In the more recent therapies several factors play a key role:

1. Identifying the cancer cell:

2. Identifying a marker which is unique on the cell:

3. Using the marker as a means to attack just those cells:

Thus the use of markers, typically endogenously created by the cell itself, is critical. But the problem often is the complexity of finding the marker. One approach is to use what are called aptamers, small oligonucleotides, single strands of DNA or RNS, 25 to say 50 nucleotides long, and using large libraries of them, say 10^{15} , to rapidly home in on a good marker. Aptamers have been shown to facilitate this process. We examine how these may be applied to the treatment of cancers.

As Cerchia has recently noted:

Aptamers, isolated by the Systematic Evolution of Ligands by EXponential enrichment (SELEX) process, are highly structured, short, single-stranded oligonucleotides that, because of their complex tridimensional shapes, interact at high affinity and selectivity with their targets. For their mode of action, aptamers are also called chemical antibodies; however, unlike antibodies, they exhibit high tissue penetration due to their small size and are poorly immunogenic if at all. Furthermore, aptamers are characterized by ready synthesis and chemical modifications designed to improve their stability, reduce toxicity, and allow conjugation with other chemical entities (including therapeutics and molecular imaging probes).

Since their first discovery in 1990, aptamers have spawned a productive academic and commercial industry. Still, the translation of pre-clinical research on aptamers into clinical use as cancer therapeutics is more advanced than generally thought, with two aptamers, the anti-nucleolin AS1411 aptamer and the anti-CXCL12 NOX-A12 aptamer, currently under evaluation as anti-cancer therapies. Researchers worldwide are attempting to the generation of aptamers to use as recognition elements in a number of applications, including cancer biomarkers discovery, targeted therapy, in vitro diagnosis and in vivo imaging modalities, and several key articles have recently appeared, indicating that this is a rapid evolving field

The key change is that we now know how to identify, with some uniqueness, a cancer cell, and the identification can, in many cases, lead to targeting of the cells. This targeting can then enable either the immune system or the action of a therapeutic or both. The key advantage os aptamers seems to be the ability to produce them with relative ease, although the costs are yet to be determined.

The objectives of this analysis are several fold. Specifically:

1. Identify the aptamers and their structures

2, Discuss the means whereby the aptamers can be produced

3. Explore the dimensions of the applications in cancers

4. Examine some specific applications of aptamers in certain cancers

2   APTAMERS

Aptamers are oligonucleotide molecules that bind to some specific target molecule on a specific cell. Simply stated, they are an assembly of say 25 to 50 nucleic acids which are capable of binding to a specific site. This is the simplest definition. How does one get these? Simply by starting with say every possible combination of A, T, C, and G, and for say 25 of these we would have 4^{25} possible sequences. We often just start with say 10^{12} of these. This pool of nucleic acids is then tested against the desired targets to which we seek them to be bound.

However in this simple approach we may have them also binding to things we may not want them to bind to. Thus if we want them bound to a PCa cell, but we do not want them binding to non-PCa cell, we first test the PCa cells, keep those that bind. Second, we then test the cells we do not want to bind, and we discard any aptamer which binds to both PCa and the "good" cells. Thus we have cells which bind PCa but not "good" cells. This then is a targeted aptamer. We must then reproduce it using say a PCR process. This yields a specific aptamer.

An additional step may be included which is not just the binding to a target cell and not binding to "good" cells, but also binding to some effector cell, namely a cell which we get to attach itself to the target via an aptamer.

As Lundstrom notes:

Since the beginning of cancer immunotherapy the goal has been to develop delivery methods for tumor-associated antigens, cytokines and other potential immunostimulatory molecules. Due to the limitations of direct administration of for instance interleukins, the possibility to employ viral and non-viral delivery systems has become attractive. Interestingly, a number of viral vectors have been evaluated in immunization studies in animal tumor models and even in clinical trials.

Among non-viral delivery vehicles the single-stranded aptamers provide an attractive alternative as they possess high specificity, selectivity and binding affinity towards their targets. In this context, aptamers can be engineered to bind to such diverse targets as proteins, cells and tissues and should be seriously considered for cancer therapy.

The above is the critical observation regarding aptamers. We have seen such an approach with CAR-T cells but we know that the costs of CAR-T exceeds $500,000 per patient per year.

Steady progress in viral vector development and especially application of oncolytic viruses has also resulted in success. A number of oncolytic adenovirus and HSV vectors have been subjected to preclinical studies and clinical trials. Particularly, an advanced-generation HSV vector optimized for oncolytic and immunomodulatory activities was after a phase III trial approved in the US and Europe for treatment of cutaneous and subcutaneous melanoma.

Aptamers have significant use. The problem is often that they can be both degraded in the body by enzymes and due to their size rapidly excreted via the kidneys. We have seen that using poly-ethylene glycol as a transport adjunct, we get a PEG aptamer which has longer staying power.

We shall discuss this later. Also besides the nucleotide sequences an aptamer scheme may also be made with a translated amino acid peptides. Our focus here is on the nucleotide versions.

The following is an example[1]. As they note:

Higher-affinity RNA aptamers to GTP are more informationally complex than lower-affinity aptamers. Analog binding studies have shown that the additional information needed to improve affinity does not specify more interactions with the ligand. In light of those observations, we would like to understand the structural characteristics that enable complex aptamers to bind their ligands with higher affinity.

Here we present the solution structure of the 41-nt Class I GTP aptamer (K(d) = 75 nM) as determined by NMR. The backbone of the aptamer forms a reverse-S that shapes the binding pocket. The ligand nucleobase stacks between purine platforms and makes hydrogen bonds with the edge of another base. Interestingly, the local modes of interaction for the Class I aptamer and an RNA aptamer that binds ATP with a K(d) of 6 microM are very much alike. The aptamers exhibit nearly identical levels of binding specificity and fraction of ligand sequestered from the solvent (81%-85%).

However, the GTP aptamer is more informationally complex (approximately 45 vs. 35 bits) and has a larger recognition bulge (15 vs. 12 nucleotides) with many more stabilizing base-base interactions. Because the aptamers have similar modes of ligand binding, we conclude that the stabilizing structural elements in the Class I aptamer are responsible for much of the difference in K(d). These results are consistent with the hypothesis that increasing the number of intra-RNA interactions, rather than adding specific contacts to the ligand, is the simplest way to improve binding affinity[2].

The figure below depicts the specific aptamer as discussed. It has a specific binding site for the GTP location.

---

[1]   AS Rose, AR Bradley, Y Valasatava, JM Duarte, A Prlić and PW Rose. Web-based molecular graphics for large complexes. ACM Proceedings of the 21st International Conference on Web3D Technology (Web3D '16): 185-186, 2016. doi:10.1145/2945292.2945324 and AS Rose and PW Hildebrand. NGL Viewer: a web application for molecular visualization. Nucl Acids Res (1 July 2015) 43 (W1): W576-W579 first published online April 29, 2015. doi:10.1093/nar/gkv402 http://www.rcsb.org/3d-view/2AU4/0

[2] http://www.rcsb.org/structure/2AU4
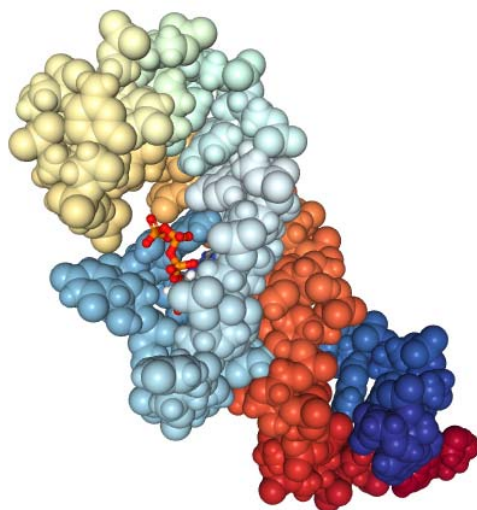
From Morita et al they note:

Aptamer-related technologies represent a revolutionary advancement in the capacity to rapidly develop new classes of targeting ligands. Structurally distinct RNA and DNA oligonucleotides, aptamers mimic small, protein-binding molecules and exhibit high binding affinity and selectivity.

Although their molecular weight is relatively small—approximately one-tenth that of monoclonal antibodies—their complex tertiary folded structures create sufficient recognition surface area for tight interaction with target molecules. Additionally, unlike antibodies, aptamers can be readily chemically synthesized and modified. In addition, aptamers' long storage period and low immunogenicity are favorable properties for clinical utility. Due to their flexibility of chemical modification, aptamers are conjugated to other chemical entities including chemotherapeutic agents, siRNA, nanoparticles, and solid phase surfaces for therapeutic and diagnostic applications.

However, as relatively small sized oligonucleotides, aptamers present several challenges for successful clinical translation. Their short plasma half-lives due to nuclease degradation and rapid renal excretion necessitate further structural modification of aptamers for clinical application. Since the US Food and Drug Administration (FDA) approval of the first aptamer drug, Macugen® (pegaptanib), which treats wet-age-related macular degeneration, several aptamer therapeutics for oncology have followed and shown promise in pre-clinical models as well as clinical trials. This review discusses the advantages and challenges of aptamers and introduces therapeutic aptamers under investigation and in clinical trials for cancer treatments.

Now aptamers are also small, as noted, and they are essential fragments of DNA or RNA or in some cases short protein sequences. This may then places them in a short life cycle since many

enzymes may recognize them and they are then attacked. Also the small size renders them susceptible to passage through the kidney and elimination by that route can be very fast.

3   IMPLEMENTATION

We now examine the means by which the aptamers can be produced. We discuss two standard means and then propose a third. It appears that aptamers can be produced more efficiently and putatively at a substantially lower cost than CAR-T cells. They can be made to have a higher in situ lifetime by use of PEG adhesion and this has already been demonstrated in the case of wet Macular Age Related Degeneration. We present three possible methods, the first two of which are in use and the third is a proposed version of a CAR-T form.

It should be remembered that aptamers are effectively homing devices, they find cells with certain surface markers and they can be loaded upon other payloads such as therapeutics to be targeted at cancer cells. The objective are:

1. Develop an aptamer to target a specific cell type and just that type.

2. Embody that aptamer on some vehicle which will allow for a sustained lifetime in the target individual.

3. Attach a therapeutic payload which will attack and destroy the target cells.

These three steps can be readily achieved in the case of aptamers.

3.1   SELEX

SELEX stands for "*Systematic Evolution of Ligands by EXponential enrichment*" since an aptamer is essentially a ligand, as a result of its production and its production is exponential via a PCR step in the cycles of the SELEX process. We now examine the literature to present the various methodologies.

From Guo:

SELEX (systematic evolution of ligands by exponential enrichment) is a method to generate DNA or RNA ligands from a combinatorial library. This oligonucleotide library consists of single stranded modified or unmodified RNA or DNA. The ligands that emerge from SELEX have been called aptamers.

Aptamers have promising advantages compared to antibodies: they can be produced easily and inexpensively and are simple to chemically modify and integrate into different analytical schemes. Aptamers can retain their binding and inhibitory behavior after immobilization on a carrier material or after delivery into animals and can be labeled with various functional groups .

From Stoltenberg et al:

Basic steps of a SELEX process are presented ...

Iterative cycles of in vitro selection and enzymatic amplification mimic a Darwinian type process driving the selection towards relatively few, but optimized structural motifs as solution for a given problem (e.g. ligand binding).

Starting point of a typical SELEX process is a chemically synthesized random DNA oligonucleotide library consisting of about 10^{13} to 10^{15} different sequence motifs. In a SELEX procedure which is directed to the selection of DNA aptamers, this library can be used without any pretreatments, whereas a conversion into an RNA library has to accomplish prior starting an RNA SELEX process.

…In either case the randomized RNA or DNA pool is incubated directly with the target. The binding complexes are subsequently partitioned from unbound and weakly bound oligonucleotides. This is one of the most crucial aspects of an aptamer selection process and strongly affects binding features of the aptamers to be selected. Target bound oligonucleotides are eluted and amplified by PCR (DNA SELEX) or reverse transcription (RT)-PCR (RNA SELEX). The resulting double-stranded DNA has to be transformed into a new oligonucleotide pool by separating the relevant ssDNA or by in vitro transcription and subsequent purifying the synthesized RNA.

This new and enriched pool of selected oligonucleotides is used for a binding reaction with the target in the next SELEX round. By iterative cycles of selection and amplification the initial random oligonucleotide pool is reduced to relatively few sequence motifs with the highest affinity and specificity for the target.
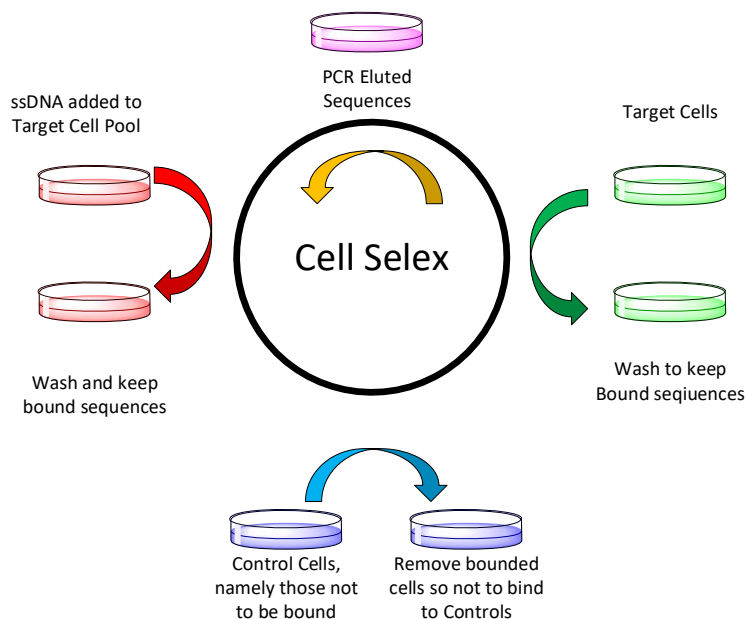
3.2 CELL-SELEX

The SELEX approach just targets a specific cell, and there may be many other cells which are targeted as well. Thus if our intent is to target a specific cell only we must first get an aptamer which targets the cell in question and then remove all aptamers which target anything BUT the desired cell. Then we get a cell specific aptamer. This approach is called the Cell-SELEX approach

From Guo we have a simple explanation:

A modification of the traditional SELEX process that uses whole living cells as target was named cell-SELEX. Aptamers with high affinity and specificity for cells have been produced successfully, demonstrating that complex targets, including tumor cells and tissues, are compatible with the SELEX process.

Using purified proteins as targets has the obvious advantage of easy control to achieve optimal enrichment during the selection process. But Cell-SELEX is favored in some special cases, when the clear marker target is unknown. Furthermore, since the target protein domain may be shielded and inaccessible on a cell surface, it reflects a more physiological condition when the protein is displayed on the cell surface rather than isolated as purified protein.,

We depict this process below.

3.3 SUPER CELL-SELEX

We introduce a third level aptamer, one not discussed in the literature. This one is Cell-SELEX with an added step. Namely we may want to target a specific cell but at the same time target something which we can attach to that cell as well. This we do the following:

1. Select an aptamer which targets the desired cell.

2. Eliminate the aptamers which target the desired cell but also other cells

3. Select an aptamer which targets the desired cell but also targets a second cell as well

4. Eliminate ant aptamers which target the undesired second cells

5. Select the residual aptamers that selective target two disparate cells.

We depict this below:
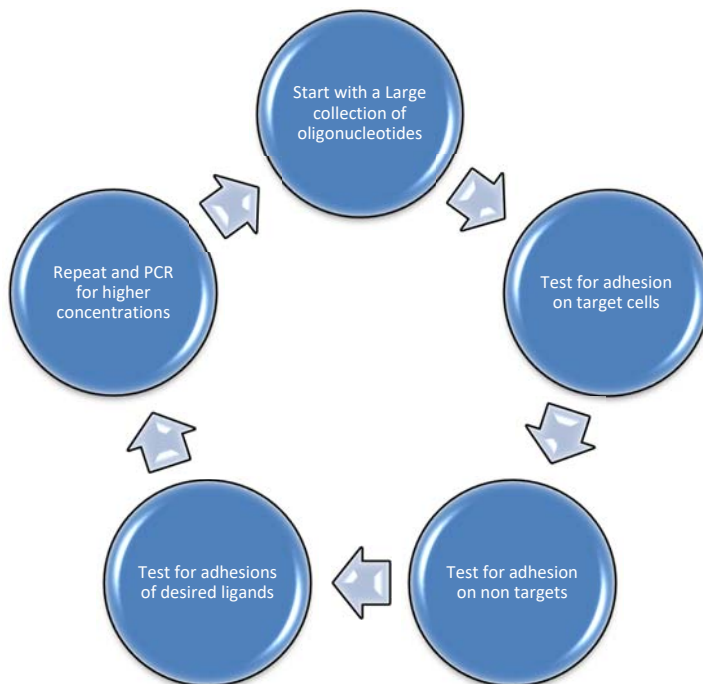
Now this third approach is intended to bring together say a targeted cell and a T cell. There is as of yet limited experimental evidence of the efficacy of this approach. In addition it may be necessary to get a larger set of aptamers so we would need a substantially larger set of starting oligonucleotides.

4   APPLICATIONS

We now examine several therapeutic applications. The literature is replete with a multiplicity of tests and trials and the ones below are but a simple example.

4.1   THERAPEUTIC DEPLOYMENT-NANOSPHERES

Aptamers are small homing devices that can target specific cells. Thus an aptamer can be used to attach itself to a desired cell and if it in turn is attached on another entity, as we discussed above in Super Cell-SELEX, then we can deliver to the target cell a lethal blow. We briefly discuss the use of nano-spheres as such an instrument.

Let us consider the following:

1. We have selected an aptamer which targets a specific cancer cell

2. We have found an aptamer that also attaches to a nano particle.

3. We create a nano-sphere, within which we embody a lethal therapeutic to the target cell.

4. We use some form of aptamer protective to ensure sustainability such as polyethylene glycol, PEG.

5. We inject the patient with the nano-spheres and they get taken up by the target cells as if they were an exosome.

6. The nanospheres are digested by the target cell releasing the therapeutic.

On paper this may work. We graphically show this below.
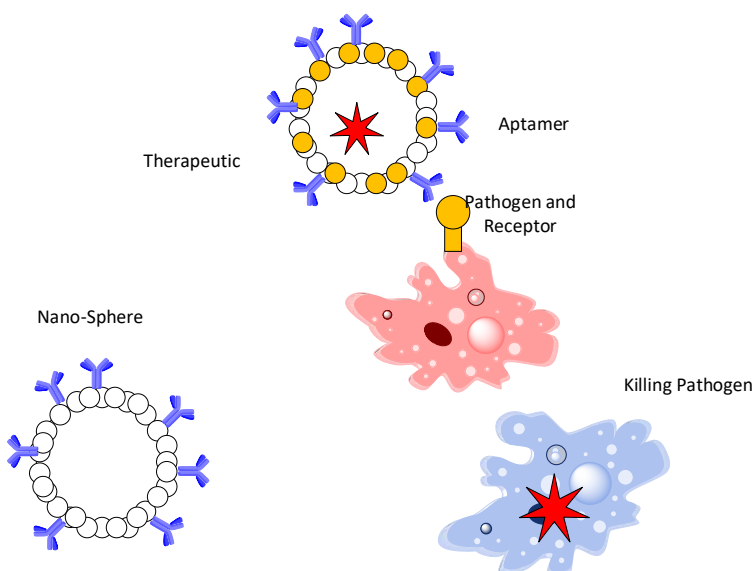
From Guo et al we know that this approach has shown viability for they note:

Applying nanotechnology to the aptamer field, Tan et al. have successfully constructed an aptamer-conjugated nanoparticle for the collection and detection of multiple cancer cells . They attached aptamers to magnetic nanoparticles (MNP) and fluorescent nanoparticles (FNP) to develop a specific platform for collecting and imaging intact leukemia cells from mixed cell- and whole blood samples. Furthermore, they developed an aptamer-conjugated nanoparticles protocol to perform the extraction of multiple cancer cell targets using additional high-affinity aptamers for recognition.

They utilized 65nm silica-coated MNP instead of the traditional micrometer-sized magnetic polymer beads. The small size and relatively high surface area of NPs provide enhanced extraction capabilities and eliminate the need for pre-sample cleanup. Upon excitation, the FNP conjugated aptamer can produce significantly higher signal intensity and -stability than other individual dye probes. The method of conjugating aptamers with nanoparticles has the potential to be used in profiling applications with further development of aptamer technology. Another new application is to fabricate electrochemical aptamers as molecular recognition elements for nanoscale biosensors. Taking advantage of small size aptamers from 10-60 bp in length, the binding with target molecules can occur inside the electrical double layer and in saline solution. By using aptamers for recognition, no labeling is required for electrochemical sensors, and signal-on architecture that is only possible with aptamers, has improved their sensitivity.

The sensors can be recycled because of the reversibility of aptamer configurations. Alternatively, bound proteins may simply be washed off without damaging the aptamers. Aptamers have already proven to be superior to antibodies in nano-sensors . Aptamer-conjugated nanoparticles for drug delivery to treat cancer cells come up as another new and exciting application. One of the biggest issues with current cancer therapies is that most of them kill both healthy and cancerous cells.

Drug delivery specialists are now presented with the challenge and the opportunity to increase the therapeutic index and ultimately deliver targeted therapies to cancer cells while minimizing off-target side effects. These bioconjugates represent an exciting prospect in the advancing field of cancer nano-technology and hold significant promise for future cancer treatment. By modifying the controlled-release polymer system or tweaking the aptamer targeting group it may be possible to produce a diverse range of specific and selective bioconjugates. In this way, drug delivery 'vehicles' could target numerous significant human cancers.

The application of nanotechnology to cancer therapy is expected to result in future therapeutic modalities that are superior to our current approach. Most importantly, this is no longer farfetched science: nanoscale drug delivery vehicles are already getting closer to clinical realization.

Thus we may have a powerful delivery system.

4.2 APTAMERS AND CANCER TARGETING

Aptamers have been examined for a variety of caner targeting. From Morita et al we have:

Aptamers as Tools for Cancer Therapy

4.2.1 RNA Aptamer Targeting VEGF

Although the VEGF targeted aptamer pegaptanib is currently approved for use in ophthalmology it was originally designed for use in cancer treatment. The cancer treatment strategy of anti-VEGF aptamer is inhibition of VEGF-associated tumor vessel formation by binding to VEGF thus blocking the VEGF/VEGF-R interaction.

4.2.2 Aptamers Targeting the Epidermal Growth Factor Receptor (EGFR)

EGFR (ErbB1) is a receptor tyrosine kinase (RTK) that is often mutated and overexpressed in many types of solid tumors, resulting in tumor growth . Thus, EGFR serves as an excellent therapeutic target, and anti-EGFR antibodies as well as TKI have been widely adopted for clinical use to block the downstream signaling cascade . Similar to anti-EGFR antibody, the binding of anti-EGFR aptamer to the extracellular domain of EGFR blocks subsequent phosphorylation and downstream signaling, i.e., PI3K/AKT and MAPK signaling

4.2.3 Aptamers Targeting HER2 and HER3

HERs including HER2 (ErbB-2), HER3 (ErbB-3), and HER4 (ErbB-4) are a part of the EGFR family and transduce growth-promoting signals in response to dimerization of extracellular domains upon ligand binding . Overexpression of HER2 is observed in approximately 10–15% of breast cancers and currently monoclonal antibody or TKI is used to block its function

4.2.4 DNA Aptamer Targeting Nucleolin

The abundant non-ribosomal protein, nucleolin, which shows abnormally increased cell

4.2.5 DNA Aptamer Targeting PD-1/PD-L1

Programmed cell death 1 (PD-1; also known as CD279) is expressed in several cell types including T-lymphocytes, specifically CD8 tumor-infiltrating lymphocytes, which are in charge of directly eradicating tumor cells . The interaction between PD-1 expressed on the surface of T-lymphocytes and PD-L1 expressed on cancer cells leads to an impairment of CD8 cytotoxicity.

4.2.6 RNA Aptamer Targeting SDF-1 (CXCL12)

CXCL12 (C-X-C motif chemokine ligand 12), also known as SDF-1 (stromal cell-derived factor-1), is a critical chemokine involved in tumor metastasis, angiogenesis, cancer cell homing, and proliferation . NOX-A12 is a PEGylated mirror-image RNA aptamer that displays high binding affinity to CXCL12 at a Kd of 0.2 nM . Through binding to CXCL12, NOX-A12 inhibits signaling

on both its receptors, CXCR4 and CXCR7, thus preventing angiogenesis as well as tumor cell proliferation, invasion, and metastasis . Based on the efficacy of NOX-A12 as a treatment in a pre-clinical study of hematologic malignancies, it is currently in clinical trials for treatment of leukemia (CLL) and multiple myeloma (see Clinical Trials section).

4.2.7   Thioaptamer Targeting E-Selectin

E-selectin is an adhesion molecule expressed on the luminal surface of inflamed blood vessels which mediates hematogenous metastasis by assisting shear-resistant adhesion of circulating tumor cells to the inflamed vessel surface under dynamic blood flow .

4.2.8   RNA Aptamer Targeting CD40

CD40Apt, a 2-fluoro-RNA aptamer against CD40, has shown antitumor effect on CD40-expressing A20 lymphoma cells in vitro and in vivo. Soldevilla et al. reported that CD40Apt-SMG1-shRNA chimera improved survival of BALB/c mice that were intravenously inoculated with A20 lymphoma cells compared to CD40Apt-control-shRNA or untreated mice .

4.2.9   DNA Aptamer Targeting CTLA-4

Monoclonal antibodies targeting cytotoxic T lymphocyte antigen-4 (CTLA-4) and PD-1/PD-L1axis are now a part of routine clinical practice . Huang et al. reported a novel high-affinity CTLA-4-antagonizing DNA aptamer that promoted lymphocyte proliferation and inhibited tumor growth in a murine syngeneic tumor model with mouse TC-1 lung cancer cells .

4.2.10 RNA/DNA Aptamer Targeting C5a

A synergistic antitumor effect through inhibition of C5a/C5a receptor-1 and PD-1 signaling has been reported. Ajona et al. showed that blockade of PD-1 via RMP1-14 antibody and signaling inhibition of complement C5a/CD5a receptor via AON-D21 L-aptamer, reduced tumor growth and metastasis in syngeneic models of lung cancer. This study further showed a complete reversal of CD8 T-cell exhaustion contributed prolonged survival in mice receiving dual therapy .

4.2.11 Thioaptamer Targeting Annexin A2

Given the central role angiogenesis plays in cancer progression, therapies have sought to target immature tumor blood vessels, yet have only achieved limited efficacy due to treatment induced-hypoxia. Mangala et al. developed a novel approach for the identification of target thioaptanmers using patient-derived endothelial cells . Thioaptamers specific to tumor associated endothelial cells isolated from ovarian cancer patients were isolated following repeated cycles of negative and positive selection.

This unbiased selection method allows for a selection of aptamers that specifically bind to tumor vessels but not to normal vessels. Mass spectrometry data identified a molecular target, the tumor endothelial cell specific membrane protein annexin A2. Treatment with the Cancers 2018, 10, 80 10 of 22 aptamer/microRNA-inhibitor complex restored tight junction function and

improved chemotherapy delivery in orthotopic ovarian cancer mouse models, reducing tumor growth.

4.2.12 Bispecific Aptamers

Bispecific aptamers have been generated to specifically and simultaneously interact with two independent targets. RNA-based bispecific CD44-EpCAM aptamer is capable of blocking CD44 and EpCAM simultaneously by fusing single CD44 and EpCAM aptamers with a double stranded RNA adaptor. Bispecific CD44-EpCAM aptamer suppressed intraperitoneal tumor outgrowth more significantly than individual CD44 and EpCAM aptamers did alone or in combination through enhanced targeting of cancer cells. Bispecific aptamers are well adapted for anti-cancer immunotherapy with minimal toxicity.

4.3 PROSTATE CANCER

There has been a significant amount of recent studies of apatemers being used in the diagnosis, prognosis and treatment of various cancers and we examine that for prostate cancer, PCa. PCa is a significant disease with a high incidence, morbidity and mortality. To date there have been a few therapeutics available and a few prognostic tests[3].

As Juan-Rivera and Martinez-Ferrer have noted:

Aptamers Inhibits α6β4 Integrins: The α6β4 integrin belongs to the group of laminin-332 binding integrins that are present in endothelial, epithelial, and Schwann cells, as well as in keratinocytes. The α6β4 integrin is the central component of hemidesmosomes, which mediate cell adhesion by connecting the intracellular keratin cytoskeleton to the cell basement membrane. Nevertheless, the β4 integrin has distinctive cytoskeletal and signaling functions via its 1017 amino acid long cytoplasmic domain. This domain can be phosphorylated by protein kinase C or by interactions with growth factor receptors that result in the release of the α6β4 integrin from hemidesmosomes .

The phosphorylation of the α6β4 integrin promotes migration and pre-metastasis signaling pathways . Recent studies with different types of cancer, such as lung cancer and colon adenocarcinoma, have shown that the inhibition of the α6β4 integrin/laminin-332 interaction reduces cell growth, invasion, and metastasis Integrin a6b4-Specific DNA Aptamer Aptamers, are short single stranded oligonucleotides with high affinity and specificity for target molecules, including proteins, peptides, metal ions, small molecules, and cancer cells, due to their unique three-dimensional folding .

They are considered targeted therapies (smart drugs) with low toxicity that selectively control cancer cell progression by inhibiting cell surface proteins. Their selection occurs in vitro through an amplification process called systematic evolution of ligands by exponential enrichment (SELEX). Since the establishment of SELEX in 1990, many aptamers have been

---

[3] We have examined these in some detail and the results are in The Telmarc web site, www.telmarc.com

generated against a variety of targets, including small chemical compounds targeting large multi-domain proteins.

To date, there are 11 aptamers in clinical trials for the treatment various diseases, such as inflammation and cancer, including PCa

As Wang et al note for PCa selection:

In this work, we have developed a new aptamer probe Wy-5a against PCa cell line PC-3 by cell-SELEX technique. Wy-5a shows high specificity to the target cells with dissociation constants in the nanomolar range, and does not recognize other tested PCa cell lines and other tested tumor cell lines. The staining of clinical tissue sections with fluorescent dye labeled Wy-5a shows that sections from high risk group with metastasis exhibited stronger fluorescence and sections from Benign Prostatic Hyperplasia (BPH) did not exhibit notable fluorescence, which suggests that aptamer Wy-5a may bind to protein related to the progression of PCa. The high affinity and specificity of Wy-5a makes this aptamer hold potential for application in diagnosis and target therapy of PCa...
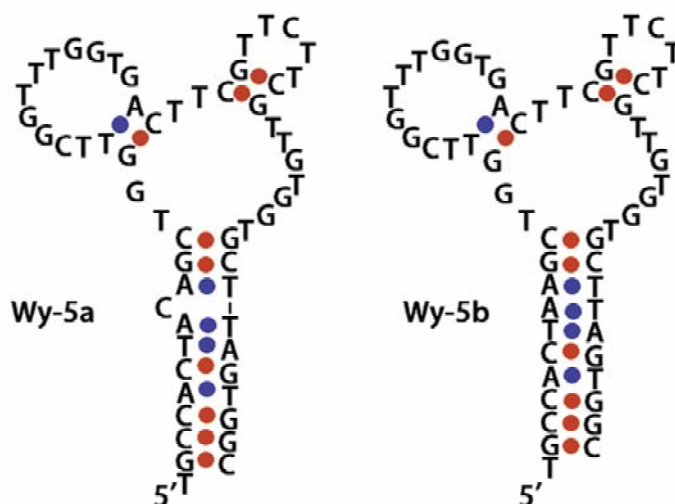
The process of aptamer selection is shown.…

The target cell line PC-3 is a typical cell line from androgen independent cancer patient with osseous metastasis. Androgen independent cancer patients with osseous metastasis are the most difficult to treat.

In order to generate aptamers with high specificity, we used more than one kind of cell line as negative control for counter selection, including nontumor-immortalized prostate epithelial cells line RWPE-1, human hepatic carcinoma cell line SMMC-7721 and Human cervical cancer cell line Hela.

For the first round selection, the target cells were incubated with the random DNA library pool.

After washing, the remained sequences on cells were amplified by polymerase chain reaction (PCR), and separated into single-stranded sequences for the second round selection.

From the second round selection, the negative cells were incubated with the enriched pool before the target cell binding in order to eliminate the sequences that bound to the common molecules present on the surface of target cells and control cells.

For every two or three rounds of selection, the aptamer enrichment was monitored by flow cytometry and confocal imaging. If aptamer sequences were enriched, the fluorescence intensity on the surface of PC-3 cells became stronger after incubation of cells with the selected pools.

As shown in Figure 2A, the fluorescence intensity on the surface of PC-3 cells greatly increased in the first ten rounds of selection, and the fluorescence enhancement on the control cells (SMMC-7721, Figure 2B) was much smaller, indicating that aptamers for target cells were greatly enriched.

However, after performed further five rounds of selection, although the fluorescence on PC-3 cells was still stronger than on control cells, the fluorescence enhancement on PC-3 cells became slower and that on control cells became faster, which suggests that more nonspecific sequences that bound to both cell lines were enriched. Confocal imaging (Figure 2C) showed that aptamers bound on the membrane of the target cells.

After another two rounds of selection with stronger counter selection, the 17th round pool was cloned and 50 clones were sequenced. Identification of aptamers against target cells.

After alignment, the obtained sequences of 50 clones were found to distribute into 5 families according to the similarities of their sequences. Through analyzing the predicted secondary structure of the sequences in each family, six sequences were truncated and synthesized for binding assay. Flow cytometry assay showed that a sequence, Wy-5a exhibited the strongest binding to PC-3 cells and the weakest binding to other cell types...; therefore this sequence was further characterized.

The secondary structure prediction showed that Wy-5a adopted a stem-loop structure with a one-base bulge on the stem.

Removing the one-base bulge, a perfect stem-loop structure sequence Wy-5b was also synthesized for further characterization. Binding assay showed that Wy-5a and Wy-5b specifically bound to PC-3 cells … The higher affinity of Wy-5a suggests that the one-base bulge on the stem of Wy-5a may involve the binding to its target. Because of the higher affinity of Wy-5a, it was further characterized as a novel probe for PCa detection.

As Tonry et al have noted:

Aptamer-Based: Despite the advances in the technologies described above in terms of sensitivity, fast response time, miniaturization, low cost and point of care applications, the availability of

antibodies for target proteins still provides a significant limitation. As such, immunosensors based on aptamer interactions are becoming a more favorable approach for sensitive detection of low molecular weight analytes of interest. Aptamers are DNA or RNA molecules with tridimensional conformation that gives them high affinity for specified biomolecules of interest.

In contrast to antibodies, aptamers can be easily modified, are smaller in size, cheaper to produce and can be generated against a wide variety of different target molecules.

Most aptamers are directly selected against the target analyte and are considered to be more sensitive than an antibody for the same analyte. In fact, problems of capture-reagent cross reactivity and non-specific adsorption to surfaces are greatly reduced with aptamer-based platforms.

As such, diagnostic/discovery approaches based on aptamers offer a robust and reliable system for detecting target(s) of interest in direct, indirect and sandwich concepts . Aptamer technology has been successfully applied for the detection of PSA in both PCa cells biopsies and human serum. With aptamer-based technology, PSA is detectable at levels as low as fg/mL with high specificity .

A modification of this platform is the SOMAscan assay, which uses slow off-rate modified aptamers (SOMAmers). These are single stranded DNA aptamers that contain pyrimidine residues carrying hydrophobic entities at their 51 position. The affinity of SOMAmers is considerably higher than that of simple RNA or DNA aptamers . Moreover, the platform is highly automated and scalable to allow for high sample throughput .

This technology is therefore considered an ideal platform for protein biomarker discovery and evaluation as it has the capacity to detect in excess of 1125 proteins in a single analysis using minimal amounts (<100 L) of serum . … the SOMAmer platform was used to quantify 1033 proteins simultaneously with sub-pM limits of detection and inter-assay CV of <5% in human serum samples. This analysis resulted in a 7-marker signature for detection of lung cancer in current and former smokers with an AUC of 0.85 for all and 0.93 for squamous cell carcinoma . This study therefore indicates the potential benefits of applying this technology for PCa-related biomarker research.

5   OBSERVATIONS

Aptamers are another form of personalized medicine.

5.1 STEM CELLS

We know that there is a stem cell construct which appears to exist in many cancers. The problem we have in any targeting is that we develop our targets on the cells which proliferate the most, and these are not the stem cells but the stem cells are the drivers of this process. Perhaps the markers on the stem cells are different than those on the proliferated cells and thus we may just be running a war on the "foot soldiers" where the "generals" are remaining unharmed.

5.2 COLLATERAL DAMAGE

Like so many therapies, even as we become more specific there is always the issue of collateral damage. CAR-T cells have "cytokine storm" effects where we get the immune system turned on too strongly. Thus one could suspect that since an aptamer is at best perceived as an antigen and would be less likely to create such a cytokine storm and therefore would reduce the risk of collateral damage. Substantial clinical trials are required, some of which are covered in the references.

5.3 MULTIPLE THERAPIES

We have become quite familiar with the need to use multiple therapeutics to battle a malignancy. Even though one therapeutic has efficacy it may be short term.

5.4 PEG ADHESION AND TRANSPORT

One of the critical issues with aptamers is that they can rapidly be excreted via the kidneys or broken down by enzymatic action. A way around that is the use of PEG, polyethylene glycol, a means used in many other areas.

As Guo et al have noted:

"As small molecules , with a half-life of minutes to hours due to nuclease degradation, aptamers can be rapidly cleaned from the bloodstream by the kidneys. The applications of unmodified aptamers are mainly focused on treating transient conditions such as blood clotting, or treating organs, for example the eye where local delivery is possible. This rapid clearance is an advantage in some applications such as in vivo diagnostic imaging, for example a tenascin-binding aptamer underdevelopment for cancer imaging. There are several modifications available, such as 2'-fluorinesubstituted pyrimidines, polyethylene glycol (PEG) linkage, etc. ..."

Likewise see Stoltenberg et al,

"Aptamers are starting to compete antibodies as therapeutic agents. Comprehensive overviews about aptamers with therapeutic applications …In the field of therapy, several aptamers for medically relevant targets will be tested regarding their application potential. The first approved aptamer with therapeutic function is the anti-human VEGF aptamer (VEGF: vascular endothelial grow factor). The PEGylated form of this aptamer was called Pegaptanib and used as the medicinal active component of the newly developed drug for the treatment of wet age related macular degeneration. "

Also see Foster et al. Finally in Morita et al they note:

"One common solution to overcome rapid renal excretion is augmentation of the aptamer's overall size through conjugation with high molecular weight moieties such as polyethylene glycol (PEG) , cholesterol, protein, multimerization, or nanomaterials. PEG is a US Food and Drug Administration (FDA) approved hydrophilic biocompatible material that has been adopted in 12 biopharmaceuticals currently marketed for human use. PEG is commercially available in a variety of sizes (0.3 to 10,000 kDa), with different terminal functional groups for chemical conjugation. For aptamer application, PEG is widely used for enlargement of the size as well as addition of stealth effect to increase their retention in circulation"

Thus PEGylated aptamers provide a viable and accepted method for retention and transport.

5.5   PRODUCTION AND COSTS

Costs of therapeutics is a growing concern. CAR-T cells is one example of this cost and it is driven by the personalization factor; namely each patient has individually developed CAR-T cells. With aptamers one may suspect a less costly yet still personalized therapeutic regime. The key reason is that one is dealing with smaller oligonucleotides and not complex T cells. Whether this proves out in actual production is open to question.

5.6   "TOOLS" AND THEIR EVOLUTION

Almost all of what can be accomplished in cancer treatment is based upon new and improved sets of tolls, and each new tool allows for added insight. For the most part it has been incremental, yet the pace is rapidly increasing. A simple example is how we have expanded in our knowledge of the immune system as a means to attack cancers. The following is an excellent example. I can recall in the late 1960s when first studying melanomas, one took drastic measures, including limb removals, in attempts to stop the progress. For many reasons, many due to late diagnosis, the efforts were futile. By the mid-1980s Rosenberg started to better understand the immune system and was applying it to melanomas, with some insight, albeit with limited success.

As Norelli et al note:

The idea of transferring ex vivo-manipulated cells of the immune system into cancer patients for therapeutic purposes, also known as adoptive cell therapy (ACT), is an idea that dates back to

the mid- 1980s, approximately ten years before the debut of targeted therapies, when Steve Rosenberg pioneered the use of lymphokine-activated killer cells in metastatic melanoma .

In thirty years, the original concept of ACT has dramatically evolved, now embracing the use of immune effectors as disparate as cytokine-induced killer (CIK) cells, tumor infiltrating lymphocytes or TILs (T cells isolated from tumor sites, expanded and re-infused back into patients), and, more recently, T cells genetically modified to express clonal T-cell receptors (TCRs) or chimeric antigen receptors (CARs).

It should be noted that we have examined CAR-T for the now classic cases of hematological cancers with CD-19 and CIK cells for use in MDS. Norelli et al continue:

Initially conceived by Zelig Eshhar, CARs are monomeric receptors usually designed by fusing the single chain fragment variable (scFv) of a tumor-reactive mAb with a transmembrane domain and one or more signaling molecules containing intracellular immunoreceptor tyrosine-based activation motifs (ITAMs). Upon CAR modification, originally with viral vectors, but more recently also with non-viral systems , T cells become cytotoxic against tumor cells expressing the antigen recognized by the mAb of origin

Now CAR and aptamer approaches hold similar features except that CAR-T use T cells which can have substantially longer lifetimes. The authors include the following Figure which depicts several diagnostic methodologies. Clearly there is an expanding appreciation for the use of aptamers
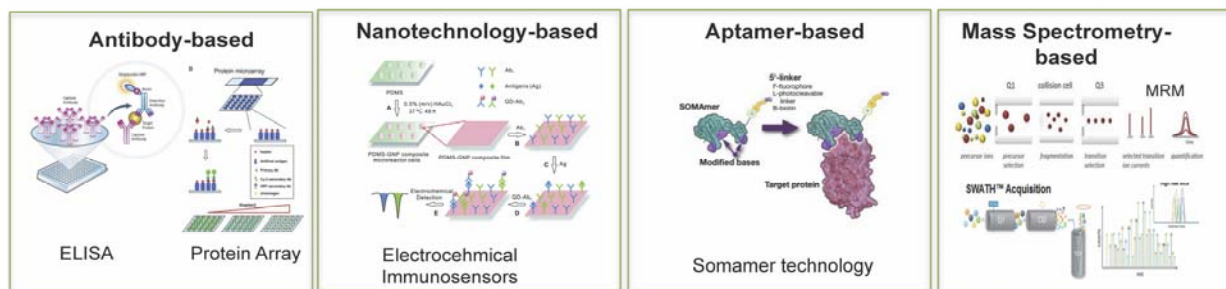
6 REFERENCES

1. Baeumler et al, Engineering Synthetic Signaling Pathways with Programmable dCas9-Based Chimeric Receptors, Cell Reports 20, 2639–2653 September 12, 2017

2. Cerchia, Aptamers: Promising Tools for Cancer Diagnosis and Therapy, Cancers 2018, 10, 132

3. Chu et al, Aptamer mediated siRNA delivery, Nucleic Acids Research, 2006, Vol. 34, No. 10

4. Farokhzad et al, Nanoparticle-Aptamer Bioconjugates: A New Approach for Targeting Prostate Cancer Cells, Cancer Research 64, 7668–7672, November 1, 2004

5. Foster et al, Improved Targeting of Cancers with Nanotherapeutics, Chapter 2, Reema Zeineldin (ed.), Cancer Nanotechnology: Methods and Protocols, Methods in Molecular Biology, vol. 1530, Springer Science and Business Media New York 2017

6. Guo et al, CELL-SELEX: Novel Perspectives of Aptamer-Based Therapeutics, Int. J. Mol. Sci. 2008, 9, 668-678

7. Hermann and Patel, Adaptive Recognition by Nucleic Acid Aptamers,

8. Hicke et al, Tumor Targeting by an Aptamer, The Journal Of Nuclear Medicine, Vol. 47, No. 4, April 2006

9. Ireson and Kelland, Discovery and development of anticancer aptamers, Mol Cancer Ther 2006;5(12). December 2006

10. Isaacs et al, RNA synthetic biology, Nature Biotechnology Volume 24 Number 5 May 2006

11. Juan-Rivera and Martinez-Ferrer, Integrin Inhibitors in Prostate Cancer, Cancers 2018, 10, 44

12. Jayasena, Aptamers: An Emerging Class of Molecules That Rival Antibodies in Diagnostics, Clinical Chemistry 45:9, 1628–1650 (1999)

13. Li et al, A Mini-Review for Cancer Immunotherapy: Molecular Understanding of PD-1/PD-L1 Pathway & Translational Blockade of Immune Checkpoints, Int. J. Mol. Sci. 2016, 17, 1151

14. Lundstrom, Oncolytic Alphaviruses in Cancer Immunotherapy, Vaccines 2017, 5, 9;

15. Morita, et al, Aptamer Therapeutics in Cancer: Current and Future, Cancers 2018, 10, 80

16. Mullis, The Unusual Origin of the Polymerase Chain Reaction, Scientific American, April 1990.

17. Norelli et al, Clinical pharmacology of CAR-T cells: Linking cellular pharmacodynamics to pharmacokinetics and antitumor effects, Biochimica et Biophysica Acta 1865 (2016) 90–100

18. Ohuchi, Cell-SELEX Technology, BioResearch Open Access, Volume 1, Number 6, December 2012

19. Stoltenburg et al, SELEX—A (r)evolutionary method to generate high-affinity nucleic acid ligands, Biomolecular Engineering 24 (2007) 381–403

20. Tonry et al, The Role of Proteomics in Biomarker Development for Improved Patient Diagnosis and Clinical Decision Making in Prostate Cancer, Diagnostics 2016, 6, 27

21. Wang et al, DNA Aptamer Evolved by Cell-SELEX for Recognition of Prostate Cancer, PLOS ONE, June 2014, Volume 9, Issue 6

22. Zboralski et al, Increasing Tumor-Infiltrating T Cells through Inhibition of CXCL12 with NOX-A12 Synergizes with PD-1 Blockade, Cancer Immunology Research, Nov 2017.