Use of Oligonucleotide Aptamer Ligands to Modulate the Function of Immune Receptors

Eli Gilboa1, James McNamara II2, and Fernando Pastor3

Abstract

The paucity of costimulation at the tumor site compromises the ability of tumor-specific T cells to eliminate the tumor. The recent U.S. Food and Drug Administration approval of ipilimumab, an antibody that blocks the inhibitory action of CTLA-4, and clinical trials targeting 4-1BB and PD-1 or PD-L1, have underscored the therapeutic potential of using immunomodulatory antibodies to stimulate protective immunity in human patients. Nonetheless, systemic administration of immunomodulatory antibodies has been associated with dose-limiting autoimmune pathologies, conceivably reflecting also the activation of resident autoreactive T cells. Arguably, targeting immunomodulatory ligands to the disseminated tumor lesions of the patient would reduce such drug-associated toxicities. We have recently developed a new class of inhibitory (CTLA-4) and agonistic (4-1BB and OX-40) ligands composed of short oligonucleotide (ODN) aptamers that exhibited bioactivities comparable or superior to that of antibodies. To reduce toxicity, the immunomodulatory aptamers were targeted to the tumor by conjugation to a second aptamer that bound to a product expressed on the surface of the tumor cell, the targeting aptamer, generating a bispecific aptamer conjugate analogous to bispecific antibodies. In a proof-of-concept study in mice, we have shown that an agonistic 4-1BB–binding aptamer conjugated to a prostate-specific membrane antigen (PSMA)–binding aptamer led to the inhibition of PSMA-expressing tumors, was more effective than, and synergized with, vaccination, and exhibited a superior therapeutic index compared with nontargeted costimulation with 4-1BB antibodies or 4-1BB aptamers. The cell-free chemically synthesized ODN aptamers offer significant advantages over antibodies in terms of synthesis, cost, as well as conjugation chemistry needed to generate bispecific ligand fusions. Clin Cancer Res; 19(5); 1054–62. ©2012 AACR.

Introduction

The review by Melero and Wolchok in this issue of Clinical Cancer Research (1) has underscored the therapeutic potential of using monoclonal antibodies to stimulate protective immunity in human patients. Nonetheless, a major limitation of using monoclonal antibodies as therapeutic agents is limited, and at best uncertain, access to this class of biologicals. The reason is that protein-based biologicals are cell-based products requiring a complex and costly manufacturing and regulatory approval process. Consequently, clinical grade antibodies are almost exclusively developed by companies and provided to academic investigators on a selective basis under strict contractual agreement and company oversight (2). Despite overwhelming evidence from murine studies attesting to the substantial synergy between 2 or more immunostimulatory antibodies, testing 2 antibodies manufactured by different companies in clinical trials is virtually impossible. A second limitation of using protein-based biologicals in human therapy is their potential immunogenicity, which can elicit neutralizing antibodies (reviewed in ref. 3). This is likely to apply also to fully humanized monoclonal antibodies and can become a limiting factor especially when repeated administrations are called for. Third, used as targeting ligands (see below), chemical conjugation of antibodies to their therapeutic cargo is complex and costly, requiring skill sets that are not readily available, especially in academic settings.

Oligonucleotide Aptamer Ligands

In this review, we will discuss an alternative platform technology of oligonucleotide (ODN) aptamer ligands (“aptamers”) to modulate the function of immune receptors. Aptamers are high-affinity single-stranded nucleic acid ligands, each specific for a given target molecule, which can be isolated through a combinatorial chemistry process using iterative in vitro selection techniques, analogous to isolation of high-affinity peptide ligands from phage display libraries. The basic approach named systematic
evolution of ligands by exponential enrichment (SELEX) depicted in Fig. 1 can be considered an extremely powerful purification method in which very rare binding activities (with frequencies of 1 in $10^{11}$–1 in $10^{13}$) are isolated by affinity purification (4, 5). Aptamers isolated by this method can exhibit remarkable affinity and specificity for their targets comparable or exceeding those of antibodies. For example, aptamers have been generated that are capable of discriminating between isoforms of protein kinase C that share a high degree of homology (6), and aptamers to coagulation factor VIIa exhibit a more than 1,000-fold higher affinity relative to coagulation factor IXa, although these proteins share a common set of structural domains (7). Aptamers can be generated using a DNA or RNA backbone; the latter being more widely used. For in vivo applications, the plasma stability of the RNA backbone of an aptamer is increased by substitution of ribonucleotides with 2'-amino, 2'-fluoro, or 2'-O-alkyl nucleotides. Accumulating experience shows that aptamers can be generated against most targets (8, 9) and can inhibit the function of the proteins to which they bind or, as we have recently shown, act in an agonistic fashion to activate receptor function (10, 11). Oligonucleotide-based aptamer ligands offer potentially significant advantages compared with that of monoclonal antibodies (and this is probably a key advantage of aptamers), the 40 to 80 nt long nuclease-resistant aptamers can be synthesized chemically. Thus preclinical development, as well as production of good manufacturing practices-grade aptamers, is more straightforward and cost-effective, and the regulatory approval process significantly simpler, to a point that a (well-funded) academic laboratory should be able to generate one to several clinical grade aptamer reagents for testing in clinical trials. Conjugation of 2 ODNs such as aptamer to siRNA or aptamer to aptamer (see below) can be accomplished in a simple and straightforward hybridization reaction using short complementary sequences appended to the ends of each ODN. Finally, though not experimentally confirmed, short ODNs are not likely to induce significant level of neutralizing immune responses.

The current protocols for isolating aptamer ligands are cumbersome and inefficient, largely responsible for the dearth of aptamer ligands against many targets of interest. New developments in this rapidly moving field, however, promise to change this. The current SELEX protocol requiring 12 to 18 rounds of selection (Fig. 1) is time consuming, costly, and often fails to identify high-affinity aptamer due to PCR bias (PCR is used after each round to amplify the target bound aptamers). In several recent studies, high-throughput sequencing enabled the identification of high-affinity aptamers after many fewer rounds of selection, reducing time, cost, and PCR bias (12–15). In the standard

Figure 1. Scheme of SELEX. A chemically synthesized combinatorial library of single-stranded ODNs, containing a 25 to 40 nt random region flanked by 15 to 20 nt constant regions used for PCR, exhibits a wide diversity of 3-dimensional structures (represented with shapes and colors in the diagram). The first step in identifying high-affinity binders to a target protein is to incubate the library with the target protein. A subset of the ODNs, which bind to the target protein, are then separated from the unbound sequences, recovered, and amplified with PCR or real-time PCR. A pool of selected single-stranded ODNs are then generated from the PCR product, and the process is repeated several times to enrich for ODNs that exhibit high-affinity binding to the target protein. For additional details, see text, refs. 4 and 5, and reviews (8, 9). Reprinted with permission from Nimjee and colleagues (9).
protocol, aptamers are selected against recombinant protein products in solution. Such aptamers often do not bind to or bind poorly to the posttranslationally modified cell surface-expressed native products. This limitation can be overcome by whole-cell SELEX whereby aptamers are selected against the native target expressed on the cell surface. For example, whole-cell SELEX was recently used to isolate a high-affinity aptamer that binds to Axl, a cell surface–expressed tumor-specific product (16). Illustrating the unique feature of this nucleic acid-based class of ligands, the in vivo bioactivity of aptamers and potential adverse effects, can be readily controlled using complementary antisense ODNs (referred to as “antidote”), as elegantly shown by reversing the anticoagulating activity of factor VIIa binding aptamers in mice and pigs (17), and recently in human patients (18). The first aptamer developed by in vitro selection targeting VEGF165 (NX-1838, named Pegaptanib sodium; Macugen) has been approved for the treatment of macular degeneration, arguably a milestone in the application of aptamer technology (19). A second aptamer targeted to the coagulation factor IXa called REG-1 is currently tested in phase I/II clinical trials to prevent blood clotting during cardiopulmonary surgery. Phase 1 clinical trials have shown an excellent safety and functional profile (18). Table 2 lists additional aptamers that are in various phases of clinical development. Readers are referred to excellent recent reviews that provide a more in depth discussion of this new platform and its therapeutic potential (20–24).

### Table 1. Main advantages of aptamers over antibodies as ligands with engineered specificity

<table>
<thead>
<tr>
<th>Advantages of aptamers compared with antibodies</th>
<th>Aptamers</th>
<th>Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simpler and more cost-effective development and manufacture of investigational and clinical grade reagents.</td>
<td>Cell-free chemical synthesis</td>
<td>Cell-based product</td>
</tr>
<tr>
<td>Straightforward conjugation to therapeutic cargo</td>
<td>Hybridization between complementary sequences</td>
<td>Complex chemistry requiring special skill sets and expensive instrumentation</td>
</tr>
<tr>
<td>Lack of, or much reduced, immunogenicity</td>
<td>Short oligonucleotides are not expected to elicit humoral responses unless tethered to a protein carrier</td>
<td>Antibodies, including humanized monoclonal antibodies, contain unique sequences and can break tolerance to common epitopes upon repeated administrations.</td>
</tr>
</tbody>
</table>

### Table 2. Clinical development of aptamers

<table>
<thead>
<tr>
<th>Aptamer</th>
<th>Target</th>
<th>Disease indication</th>
<th>Company</th>
<th>Clinical development</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macugen</td>
<td>VEGF-165</td>
<td>Macular degeneration</td>
<td>Pfizer/Eyetech Pharmaceuticals&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Market</td>
</tr>
<tr>
<td>E10030</td>
<td>PDGF-B</td>
<td>Macular degeneration</td>
<td>Ophthotech Corp</td>
<td>Phase II</td>
</tr>
<tr>
<td>REG-1</td>
<td>Factor IXa</td>
<td>Coronary artery bypass</td>
<td>Regado Bioscience</td>
<td>Phase II</td>
</tr>
<tr>
<td>ARC19499</td>
<td>TFPI</td>
<td>Percutaneous coronary intervention</td>
<td>Baxter International Inc/Archemix Corp.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Phase II</td>
</tr>
<tr>
<td>AS1411</td>
<td>Nucleolin</td>
<td>Acute myelogenous leukemia</td>
<td>Antisoma</td>
<td>Phase II</td>
</tr>
<tr>
<td>NOX-E36</td>
<td>CCL2/MCP1</td>
<td>Renal cell carcinoma</td>
<td>NOXXON Pharma</td>
<td>Phase II</td>
</tr>
<tr>
<td>NOX-A12</td>
<td>SDF-1α</td>
<td>Renal impairment</td>
<td>NOXXON Pharma</td>
<td>Phase I</td>
</tr>
<tr>
<td>NOX-H94</td>
<td>Heparin</td>
<td>Anemia of chronic disease</td>
<td>NOXXON Pharma</td>
<td>Phase I</td>
</tr>
</tbody>
</table>

Abbreviations: PDGF, platelet-derived growth factor; TFPI, tissue factor pathway inhibitor.<br><sup>a</sup>Eyetech Pharmaceuticals was acquired by Pfizer.<br><sup>b</sup>Archemix Corp. was acquired by Baxter International Inc.
their in vivo nuclease resistance as well as reduce their off-target immunostimulatory effects. The monovalent aptamer bound to its target in solution, but not to the closely related CD28 receptor, with an approximate $K_d$ of 30 nM, that compares favorably with the binding avidity of bivalent antibodies. The original 80 nt long ODN backbone was successfully truncated to 35 nt, which is not always feasible, with minimal loss of function. The aptamer was able to reverse CTLA-4–mediated inhibition of T-cell proliferation in vitro, and tetravalent CTLA-4 aptamer derivatives potentiated protective immunity in murine tumor models with comparable potency to CTLA-4 antibodies.

A second immune receptor that we chose to target with aptamers was 4-1BB, the major costimulatory receptor expressed on activated CD8$^+$ T cells (11). The goal was to develop 4-1BB aptamer ligands that costimulate CD8$^+$ T cells and potentiate (vaccine-induced) immunity. The CTLA-4–binding aptamer, and all other previously described aptamers, blocked the function of their cognate targets. The objective here was, however, to develop an agonistic 4-1BB aptamer that functions as the target’s natural ligand, namely to initiate a signaling cascade that leads to the enhanced survival of the CD8$^+$ T cells. Because receptor signaling, including signaling by 4-1BB, often requires the ligand-induced oligomerization, it was not unexpected that the monovalent 4-1BB aptamers isolated by the standard SELEX protocol bound to 4-1BB on the cell surface but did not induce 4-1BB costimulation. To convert the 4-1BB–binding aptamer into agonistic ligands, we generated bivalent 4-1BB aptamers by annealing 2 monomeric aptamers that were modified with 21 nucleotide 3’-end complementary sequence extensions (Fig. 2A). On a molar basis, the systemically administered bivalent dimeric 4-1BB aptamers were at least as effective as an anti-4-1BB monoclonal antibody (of immunoglobulin G subtype and hence bivalent by nature) at inducing the rejection of preimplanted tumors in mice (Fig. 2B). Using a similar approach, we have also shown that dimeric OX40-binding aptamers were as effective as an anti-OX40 antibody in costimulating T cells in vitro and enhancing vaccine-induced protective immunity in mice (10). Figure 3 shows the sequence and computer-generated secondary structure of the CTLA-4, 4-1BB, and OX40 aptamers discussed above, as well as a recently developed aptamer that binds to and blocks the function of murine interleukin (IL)-10 receptor (12).

In summary, aptamer ligands can be generated that bind to and modulate the function of immune receptors expressed on the surface of immune cells. Aptamers can be used to inhibit the function of receptors, presumably by blocking access to their natural ligands, or to activate the receptor in lieu of the natural ligands. It is noteworthy that the first generation of immunomodulatory aptamers that were developed to date targeting CTLA-4, 4-1BB, and OX40 were not less effective than the corresponding antibodies in terms of immune function and therapeutic impact in mice. There can be little doubt that optimizing the selection protocol to generate aptamers with significantly higher-binding affinity, as well as chemical modifications and/or conjugation to carriers to enhance their in vivo bioavailability, will further enhance their bioactivity and therapeutic potential.

Aptamers as Targeting Ligands to Modulate the Function of Immune Cells

Dose-limiting toxicity of cancer drugs is a main hurdle facing the development of effective treatments for cancer. Immunostimulatory drugs are no different. Ipilimumab, a monoclonal antibody that blocks the function of the coinhibitory receptor CTLA-4, is the first U.S. Food and Drug Administration–approved immunostimulatory antibody that has shown clinical benefit in patients with cancer. Nonetheless, ipilimumab treatment is associated with significant dose-dependent toxicities and grade 3 to 4 adverse effects including colitis and hypophysitis (26). Likewise, treatment with an agonistic 4-1BB antibody while showing antitumor efficacy was also associated with grade 3 or higher neutropenia and elevated liver enzymes (27), and severe hepatic toxicity at higher doses that led to the suspension of the clinical trial (27, 28). The potential toxicity of immunostimulatory antibodies was underscored by unexpected severe toxicities seen in healthy volunteers treated with a super agonistic anti-CD28 antibody that was not predicted by nonhuman primate studies (29). That treatment with immunomodulatory monoclonal antibodies can elicit adverse effects that did not come as a surprise. A host of autoreactive T cells that escape thymic elimination are kept in check in the periphery, reflecting an evolutionary selected balance between immune-activating processes resulting from the presentation of self-antigens in the periphery and regulatory mechanisms such as foxp3+ regulatory T cells that dampen their immunoreactivity below a threshold to cause pathologic damage. Arguably, systemic administration of immunomodulatory ligands such as antibodies or aptamers can and will perturb this balance enhancing autoreactivity and consequently the risk of adverse autoimmune pathology. Arguably, such adverse effects are likely to increase when immunomodulatory agents are used in combination. Thus, given the promise of immunostimulatory agents, ways to reduce their undesirable effects while maintaining their therapeutic impact would enhance their therapeutic index and are therefore highly desirable. The issue is how to stimulate tumor-specific T cells while at the same time limit the activation of autoreactive T cells.

One way to do that is to restrict costimulation to the tumor site. This can be achieved by targeting the immunostimulatory ligands to the disseminated tumor lesions of the patient and thereby reducing their access to autoreactive T cells. The challenge is how to accomplish this in a clinically feasible, cost-effective, and broadly applicable manner. An immediate choice is to use antibodies as targeting ligands conjugated to an immunostimulatory antibody such as anti-CTLA-4 or anti-4-1BB antibody. The tumor-targeting antibody would recognize a product that is preferentially, not necessarily exclusively, expressed on the surface of tumor cells, such as prostate-specific membrane antigen (PSMA)
expressed on prostate tumor cells or Her2Neu expressed on breast cancer cells. Yet, as discussed above, the challenges of developing clinical grade antibodies, let alone heterobispecific antibody conjugates, are probably the reason why this apparently obvious strategy has not been attempted so far. An alternative approach discussed below is to use aptamers, both as targeting and immunostimulatory ligands.

To test the feasibility of using aptamers to target costimulation to the tumor site, we generated a bispecific aptamer conjugate whereby a targeting aptamer (an aptamer that binds to human PSMA) was conjugated to a dimeric 4-1BB–binding aptamer discussed above (ref. 30; Fig. 4A). Because the PSMA-binding aptamer did not cross-react with murine PSMA, we transfected murine tumor cell lines with...
The tumor-targeted nature of the antitumor effects was shown by showing that the ΔPSMA-4-1BB aptamer conjugates homed preferentially to ΔPSMA-expressing tumors and inhibited their growth, but not to contralaterally implanted parental tumor cells, which did not express ΔPSMA.

The underlying premise of tumor-targeted immunostimulation is that it will enhance the therapeutic index of the drug, and thereby reduce the risk of adverse effects resulting from the activation of autoreactive T cells. In addition, targeting will also reduce the dose needed to elicit a therapeutic effect that will enhance the cost-effectiveness of the treatment and reduce the risk of nonspecific immunostimulation of the nucleic acid-based drug. Indeed, on a molar basis, approximately 10-fold less ΔPSMA-4-1BB aptamer conjugates was needed to elicit an antitumor response compared with 4-1BB antibody or unconjugated 4-1BB aptamer (30). As shown in Fig. 5, administration of doses that elicit comparable antitumor effects, 4-1BB antibody, but not aptamer conjugate, elicited nonspecific immunoresponses in the treated mice. This is suggestive, but by no means compelling, evidence that tumor-targeted costimulation will exhibit a favorable therapeutic index compared with that of nontargeted antibodies, the current gold standard.

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standard. Interestingly, as can be seen in Fig. 5, nontargeted administration of unconjugated 4-1BB aptamer, which like the antibody was used at 10-fold higher dose, failed to elicit nonspecific immunity. It is tempting to speculate that this is due to a shorter circulation half-life of the aptamer in the serum compared with that of antibodies, and that it is the persistence of the antibody ligand in the circulation that is responsible for the enhanced activation of autoreactive T cells; another potential advantage of aptamers over antibodies.

In summary, the studies with the ΔPSMA-4–1BB aptamer conjugate carried out in preclinical murine tumor models have provided proof-of-concept that tumor-targeted costimulation using aptamer-targeted aptamer ligands is capable of potentiating tumor immunity, is superior to nontargeted antibody or aptamer ligands, and importantly, may exhibit a superior therapeutic index. If these findings can be translated to the human experience, it means that it will be possible to escalate the intensity of costimulation with ligands to immune receptors such as CTLA-4 or 4-1BB and/or use combinations of, without running the risk of eliciting unacceptable levels of autoimmune pathologies.

Nonetheless, the main current limitation of the tumor-targeted costimulation strategy stems from the fact that the
therapeutic cargo, the costimulatory (aptamer) ligand, has to be displayed on the surface of the tumor cells. Consequently, one is limited to targeting receptors on the surface of tumor cells that do not internalize upon ligand bindings. Although receptor–ligand interactions exist, for example rituximab, an anti-CD19 antibody that kills CD19-expressing tumor cells via antibody-dependent cell-mediated cytoxicity (32), most receptor–ligand complexes are internalized, and consequently the targeting choices are significantly limited, thereby complicating the development process and its clinical use.

Where We (Can) Go from Here

Keeping in mind that the immunostimulatory agents used on these murine studies represent first generation aptamer and aptamer conjugates, and that treatment protocols used to evaluate their antitumor effects in mice have not been optimized, we may be witnessing the tip of the iceberg of the potential of using aptamer ligands as immunostimulatory drugs (not counting the ease and cost-effectiveness of development and manufacture as discussed above). This calls for developing aptamers to other immunomodulatory targets such as agonistic aptamers to CD27, blocking aptamers to Fas ligand, PD-1/PD-L1 or, as we have recently described, aptamers that inhibit the function of the IL-10 receptor (12). The aptamers can be targeted to both tumor and the immune system by conjugation to targeting aptamer, or use the aptamers as targeting ligands to deliver biologicals to cells expressing the cognate receptors. For example, aptamers to IL-10 receptor could be used to deliver therapeutic siRNAs to potentiate immunologic functions in IL-10 receptor-expressing T cells and dendritic cells. ODN aptamers, for the reasons discussed above, could also offer advantages in replacing the antibody moieties in bispecific T-cell engagers, bispecific ligands that juxtapose effector T cells, independent of T-cell receptor specificity, to tumor cells, that have shown significant therapeutic promise in clinical trials (33).

There is no “magic bullet” in cancer therapy, and that also pertains to the immunologic modality. Effective costimulation, whether using antibodies or aptamers, will be one important component in a multimodality immune-based treatment that will also need to include ways to activate the immune system and counter tumor-induced immune evasion. For example, we have recently described an alternative approach to elicit an antitumor immunoresponse by expressing new antigenic determinants in the disseminated tumor lesions using aptamer-targeted RNAi to inhibit the nonsense-mediated mRNA decay (NMD) process in tumor cells (34). Not surprisingly, 4-1BB costimulation with 4-1BB aptamer ligands synergized with the aptamer-siRNA NMD inhibition approach in potentiating tumor immunity (34).

ODN aptamer ligands are a new and emerging drug and drug delivery platform that can have a transformative impact in medicine, not limited to immunotherapy. Promising as they may be, the clinical translation of preclinical proof-of-concept studies is facing serious challenges. Key among them is the ability to isolate high-affinity aptamer ligands against the human targets, as well as the development of cost-effective protocols for manufacturing clinical grade reagents. At present, chemical synthesis of ODNs with 2′-fluoro–modified ribonucleotides above 60 to 80 nt long is becoming exponentially expensive and cost-prohibitive. The list of clinical trials with aptamers (Table 2) is, however, a testament that it is feasible. Given that the aptamer technology is in its infancy, there is reason to expect that increased awareness and interest in this platform will result in quantum leaps facilitating aptamer development and establishing cost-effective manufacturing protocols.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: E. Gilboa

Development of methodology: E. Gilboa, F. Pastor

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Gilboa, F. Pastor

Writing, review, and/or revision of the manuscript: E. Gilboa, F. Pastor, J. McNamara

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