From selection hits to clinical leads: progress in aptamer discovery

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Aptamers were discovered more than 25 years ago, yet only one has been approved by the US Food and Drug Administration to date. With some noteworthy advances in their chemical design and the enzymes we use to make them, aptamers and aptamer-based therapeutics have seen a resurgence in interest. New aptamer drugs are being approved for clinical evaluation, and it is certain that we will see increasingly more aptamers and aptamer-like drugs in the future. In this review, we will discuss the production of aptamers with an emphasis on the advances and modifications that enabled early aptamers to succeed in clinical trials as well as those that are likely to be important for future generations of these drugs.

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INTRODUCTION TO SELEX AND IN VITRO SELECTION

Whether referred to as “SELEX” (Systematic Evolution of Ligands by Exponential Enrichment) or as “in vitro selection” (a demarcation resulting from whether the technique was learned from Tuerk and Gold1 or Ellington and Szostak,2 respectively), the fundamental approach to acquiring novel aptamers remains largely unchanged despite alterations including cell targeting, cell internalization, and even in vivo selections3–7. The core of the selection process, be it for RNA or DNA aptamers, on protein, on cells, or in animals, entails essentially three critical steps: (i) incubation of a library of randomized molecules with a target, (ii) partitioning bound from nonbound species, and (iii) recovering and amplifying the bound species. The process is then repeated until the population is enriched for species with the desired function (Figure 1).

For the most part, progress in the aptamer field has not changed this fundamental approach. However, several key advances have significantly changed how aptamers can be identified from the selection, the types of targets that can be probed, and the chemistries that aptamers can incorporate—all of which leads to more expanded uses for aptamers, both in vitro and in vivo. Advances in sequencing technologies and computational analyses now provide greater insight into population dynamics during selections, potentiating the identification of functional molecules from earlier rounds of selection. New polymerases are now available that can yield expanded uses for aptamers, both in vitro and in vivo. Advances in understanding the relationship between aptamer structure and function has led to the development of optimized aptamers with desired properties.

For R&D purposes, minimized aptamers 20 to 50 nucleotides in length can be generated in individual lab using “lab scale” DNA or RNA synthesizers (e.g., Expedite 8909, ABI394) or by one of a number of the many oligonucleotide synthesis companies. Depending on oligonucleotide length and the modifications present, synthesis at the 1 μmole scale typically yields ~1 mg of material, which is often sufficient for hundreds of in vitro experiments and enough to perform preclinical analyses in small animal (mouse) models. These
Figure 2a) are highly susceptible to nuclease degradation, with the modifications, deoxyribonucleic (2’H) and ribonucleic acids (2’OH) are in the process of undergoing clinical evaluation. Without these against endogenous, ubiquitous nucleases has been instrumental. The advent of chemistries to stabilize aptamers (and nucleic acids) chemistry. Limitations of nonmodified aptamers

molecules are capable of scaling to 10–15 µmole, but for larger synthesizes, equipment capable of generating gram scale quantities of aptamers can be readily obtained (e.g., the ÄKTA oligopilot plus 100 can scale from 250 µmole to 4 mmoles). Of course, synthesis ranging from 50 nmoles to 4 mmoles and beyond can also be outsourced to a number of commercial sources that offer large-scale R&D grade materials. When advancing to clinical evaluation, adherence to current good manufacturing practice must be employed (reviewed in ref. 13). Fortunately, there are now many companies that provide this service with synthesis facilities capable of kilogram scale production. However, while the exact synthesis method for clinically evaluated aptamers, to our knowledge, are not publicly available, the synthesis methods all rely on standard solid phase phosphoramidite chemistry. Limitations of nonmodified aptamers

The advent of chemistries to stabilize aptamers (and nucleic acids) against endogenous, ubiquitous nucleases has been instrumental to the advancement of aptamers that already have, or currently are in the process of undergoing clinical evaluation. Without these modifications, deoxyribonucleic (2’H) and ribonucleic acids (2’OH) (Figure 2a) are highly susceptible to nuclease degradation, with the former displaying serum half-lives of ~1 hour16 and the latter lasting only mere minutes.17 Perhaps just as important, many of these chemistries also render aptamers nonimmunogenic.18–20 This is a significant problem for nonmodified RNA and DNA, which can activate innate responses though interaction with Toll-like receptors, in particular Toll-like receptors 7, 8, 9, and 3 (refs. 21–23). Thus, a number of different strategies and chemical modifications are now available to both enhance the stability of aptamers and render them less immunogenic.

Backbone modifications

Sugars. Modifications to the sugars such as 2’-fluoro (2’F) or 2’-amino (2’NH2) ribose groups (Figure 2a) on the pyrimidine residues have been available for incorporation into enzymatically derived nucleic acids for some years using a mutant of T7 RNA polymerase bearing a tyrosine to phenylalanine mutation at position 639 (Y639F; ref. 24). While both are effective at improving serum half-life, 2’F modifications quickly garnered favor over 2’NH2, due to entropic gains in binding caused by reduced backbone flexibility, increased coupling efficiency during solid-phase synthesis, and elimination of extra deprotection steps during 2’NH2 purification.25 The more bulky 2’-O-methyl (2’OMe) modifications (Figure 2a), while incompatible with the Y639F mutant, have been previously used as a postselection modification due to their increased nuclease resistance and higher duplex melting temperature as seen in the clinical examples below. However, more recent advances in polymerase evolution give hope for more prominent use of 2’OMe modifications at the outset of the selection process (see “New RNA Polymerase Variants” below). Another advance in nuclease stabilization is conformationally restricted sugars, or locked nucleic acids, which covalently bridge the 2’ and 4’ ribose positions (Figure 2a).26,27 Despite their enhanced stability, the conformational constraints imposed by locked nucleic acids may relegate such modifications to paired regions and stems, although here again, recent advances in polymerases now allow for their incorporation during the selection process (see “New RNA polymerase variants” below).

Phosphate modifications. Stabilizing backbone modifications can also be incorporated into aptamers by substituting backbone phosphate linkages (PO) with sulfur-containing phosphorothioate linkages (PS)28 and, more recently, phosphorodithioate linkages29 (Figure 2b). However, in the case of PS linkages, which are chiral (the PS linkage can exist in the Sp or the Rp configuration), care in placement needs to be exercised as chemical synthesis results in a mixture of enantiomers that could adversely affect function.

Capping modifications

In addition to sugar and backbone modifications, therapeutic aptamers are typically modified in two additional ways. First, the 3’ end of the molecules are usually capped with an inverted dT residue, which provides resistance to serum exonucleases (Figure 2c).18 Second, the molecules are conjugated at the 5’ end to a 40 kDa PEG moiety to slow renal clearance. Indeed, without this moiety, as a result of their small size (10–20 kDa), aptamers are readily cleared by glomerular filtration with a half-life of minutes50,51 similar to that observed for comparatively sized siRNA.32 While this can be slowed by the addition of hydrophobic moieties such as cholesterol or diacyl lipids,30,31,33,34 the addition of PEG has proven to be the most useful and commonly pursued approach, slowing clearance half-lives to 3–50 hours depending on the PEG size.30,31,35–37 The ability to chemically synthesize aptamers affords a significant amount of freedom in the design of conjugation schemes for any chemical moiety. For example, amine, thiol, aminooxy, alkyne, aldehyde, and azide functional groups can all be incorporated into the aptamer during solid phase synthesis (Figure 2e). For small, lipophilic modifications (e.g., cholesterol, palmitate) incorporation can be readily achieved using a phosphoramidite derivative of the molecule. However, for the larger hydrophilic moieties (e.g., PEG 20 or 40 kDa), conjugation is typically achieved post-synthesis via the reaction of a 5’ amine, introduced on the aptamer using a hexylamine linker (Figure 2e; i) and an N-hydroxysuccinimide-derivative of the polymer.

CLINICALLY EVALUATED APATRAMERS: PAST AND PRESENT

Aptamers evaluated in phase 3 trials

Pegaptanib. The anti-vascular endothelial growth factor (VEGF) aptamer, pegaptanib (Macugen), originally selected by NeXstar Pharmaceuticals (later taken on by Ophthotech) for the treatment...
of neovascular (wet) age-related macular degeneration, remains the only US Food and Drug Administration-approved aptamer to date.\(^{38}\) Originally selected from a 2’F pyrimidine RNA library, the lead candidate, clone t44 bound VEGF\(_{165}\) with \(\sim 10\) pmol/l affinity (Figure 3-t44 and Table 1), though showing no measurable affinity for the VEGF\(_{121}\) isoform.\(^{25}\) For added stability, the 2’OH purines were systematically replaced with 2’OMe purines, and all but two could be converted with binding affinity only rising to 50 pmol/l. The molecule was then further modified with a 3’ inverted dT to inhibit 3’ exonuclease activity and a branched 40 kDa PEG via a 5’ terminal amine to slow clearance (Figure 3-pegaptanib). In its fully modified form, the aptamer is reported to have a serum degradation half-life of 18 hours (Table 1).\(^{39}\) This is even longer in the eye where the molecule is only turned over due to plasma clearance, and, after a single 0.5 mg injection in rhesus monkeys, sees a half-life of 2 days with detectable amounts of intact aptamer for more than 28 days.\(^{40}\) In humans, a half-life of \(\sim 10\) days has been reported.\(^{41}\) Approved in 2004, Macugen was the first US Food and Drug Administration-approved aptamer and also the first anti-VEGF drug, though it is no longer widely used given its limited isoform recognition compared to its protein competitors Avastin, Lucentis, and Eylea.\(^{42–44}\)

**Figure 2** Modifications utilized to enhance the in vivo stability of aptamers. (a) 2’-modifications can easily be incorporated into aptamers during chemical synthesis and include i. 2’H, ii. 2’OH, iii. 2’NH\(_2\), iv. 2’F, v. 2’OMe and vi. locked nucleic acids. (b) Increased stability can also be garnered through thiolation of the phosphate backbone. Structures shown (from left to right) are for the i. natural phosphodiester, ii. the thiolated phosphotheioate and iii. phosphorothioate. (c) 3’ inverted deoxythymidine residue. (d) Non-ribose backbones, which can be incorporated using novel DNA polymerases for the basis for xeno nucleic acids, include i. cyclohexenyl, ii. arabinio, iii. \(\alpha\)-L-threofuranosy, and iv. 2’-fluororarabinio nucleic acids. (e) Examples of some commercially available functional groups that can be readily attached to the 5’-end during solid phase synthesis and used to facilitate downstream conjugation include i. amine, ii. alkyne, iii. azide, iv. thiol, v. aldehyde and vi. aminooxy.

**Pegnivacogin.** Similar to Macugen, the Factor IXa aptamer pegnivacogin, which prevents blood coagulation, arose from a selection with a 2’NH\(_2\) pyrimidine RNA library.\(^{28}\) While these molecules did not advance to clinical evaluation, the study did provide important insight regarding how post-selection stability enhancements can be used to stabilize aptamers and prepare them accordingly for clinical evaluation. For example, while the lead candidate from this selection, NX-107, displayed a degradation half-life in mouse urine (a nuclease rich surrogate for serum) of \(\sim 1.4\) hours, the addition of PS caps or a combination of PS caps and 2’OMe purine substitution dramatically increased degradation half-lives to 17 and 131 hours, respectively (Table 1).\(^{28}\) It is worth noting that the same authors who reported t44 also performed an earlier VEGF selection with a 2’NH\(_2\) pyrimidine RNA library.\(^{28}\) While these molecules did not advance to clinical evaluation, the study did provide important insight regarding how post-selection stability enhancements can be used to stabilize aptamers and prepare them accordingly for clinical evaluation. For example, while the lead candidate from this selection, NX-107, displayed a degradation half-life in mouse urine (a nuclease rich surrogate for serum) of \(\sim 1.4\) hours, the addition of PS caps or a combination of PS caps and 2’OMe purine substitution dramatically increased degradation half-lives to 17 and 131 hours, respectively (Table 1).\(^{28}\)
is only half of the Reg1 Anticoagulant System, and is designed to work in conjunction with anivamersen, a complementary (antisense) 2’OMe oligonucleotide, which hybridizes with and inactivates pegnivacogin to quickly reverse the anticoagulation effect, representing a breakthrough in controllable drug design.\textsuperscript{47} Tested initially in both porcine\textsuperscript{48} and baboon models,\textsuperscript{49} the systemically delivered, PEGyated, and backbone modified aptamer demonstrated high \textit{in vivo} stability with a duration of action of over 30 hours with a 60 mg injection in humans,\textsuperscript{50} but could be inactivated following i.v. administration of the antisense “antidote” within minutes.\textsuperscript{51,52} The aptamer could also be delivered subcutaneously to provide sustained release over a period of days.\textsuperscript{53} The REG1 Anticoagulation System made it as far as phase 3 before a combination of severe allergic reactions, which were observed in 10 of 1,605 patients (including one fatal reaction) and the absence in any difference in the primary endpoint between patients receiving REG1 or the control group, which received bivalirudin, halted the trial.\textsuperscript{54} While the lack of improved efficacy of REG1 when compared to bivalirudin may be a consequence of the study design,\textsuperscript{55} more alarming to the aptamer community are safety concerns. However, recent analysis\textsuperscript{56} of existing samples from the RADAR\textsuperscript{57} phase 2b study indicated that severe allergic reactions in patients during that trial were correlated to high levels of pre-existing anti-PEG antibody and \textit{not} caused by the aptamer itself;\textsuperscript{56} good news for the field. Given the widespread use of PEG on the aptamers mentioned in this review (Table 1), the investigation into alternative moieties for avoiding renal clearance may prove important to the field.

\textit{Pegpleranib.} The most recent aptamer to make significant progress in clinical trials is Fovista, commonly called pegpleranib (formerly E10030), an aptamer that targets platelet-derived growth factor. Fovista is currently under development by Ophthotech and is undergoing simultaneous phase 2/3 clinical trials. The aptamer, like Macugen, is designed to be delivered via intravitreal injection and is intended to work synergistically with VEGF inhibitors, such as Macugen, Avastin, Lucentis, and Eylea in the treatment of age-related macular degeneration. Unlike Macugen and Reg1, however, Fovista was selected from a ssDNA library with a minimized lead molecule, 36t, displaying an affinity of \(\sim100\) pmol/l for the AB and BB isoforms (Figure 3-36t).\textsuperscript{58} After validation as a ssDNA aptamer, which had a serum degradation half-life of \(\sim0.6\) hours, Fovista was back-filled with both 2’F pyrimidines and 2’OMe purines, increasing the serum half-life to \(\sim8\) hours while maintaining \(\sim100\) pmol/l affinity.\textsuperscript{59} Additionally, loop regions within the aptamer found to be functionally unimportant were dispensed of in favor of short, PEG linkers, which likely not only added to the stability, but decreased overall cost of production by minimizing the number of nucleotides in the molecule (Figure 3-pegpleranib).\textsuperscript{59}
Though demonstrably effective in their clinical evaluations, one common feature between Macugen, pegnivacogin, and Fovista is that their original selections were all published in the first 12 years of this now 25-year-old field. One is thus left to wonder where new clinical candidates are, and what they look like if they harness the advancements of the last 13 years of progress.

### Table 1: Summary of characteristics for aptamers discussed in the text

| Target  | Aptamer  | 2’ Pyrimidine | 2’ Purine | 5’ mod | 3’ Mod | Other | $K_d$ (pmol/l) | Stability $t_{1/2}$ (hours) | Clearance $t_{1/2}$ (α, β) (hours) | Phase | Status | Ref. |
|---------|----------|----------------|-----------|--------|--------|-------|---------------|-------------------------------|-------------------------------------|--------|--------|------|
| Anti-VEGF | t44      | F              | OH        | --     | --     | --    | 10            | --                            | --                                      | --     | --     | 25   |
| Pegaptanib | F        | OMe/OH        | PEG 40k   | 3'-3' dT   | -- | -- | 50          | 18; HP | ~240; H (ivt) | 3 | Approved | 25,39,41 |
| NX-107 | NH$_2$ | OH | -- | -- | -- | -- | NR | 1.4; MU | -- | -- | 28 |
| NX-178 | NH$_2$ | OH | PS cap | PS cap | -- | 2,400 | 17; MU | -- | -- | 28 |
| NX-213 | NH$_2$ | OMe/OH | PS cap | PS cap | -- | 140 | 131; MU | -- | -- | 28 |
| ARC245 | OMe | OMe | -- | -- | -- | 1,000 | -- | -- | -- | -- | 80 |
| Anti-Factor Xa | 9.3t | F | OH | -- | 3'-3' dT | -- | 600 | -- | -- | -- | -- | 45 |
| Peg-9.3t | F | OOH | PEG 40k | 3'-3' dT | -- | 3,000 | -- | -- | -- | -- | 45 |
| Pegnivacogin | F | OMe/OH | PEG 40k | 3'-3' dT | -- | 3,000 | -- | >30; H (iv) | ~144; H (sc) | 3 | Terminated | 50,53 |
| Anti-PDGF | 36t | H | H | -- | 3'-3' dT | -- | 100 | 0.6; RS | -- | -- | -- | 58,59 |
| Pegpleranib | H/F | H/OMe | PEG 40k | 3'-3' dT | -- | 100 | 8; RS | -- | 3 | Ongoing | 59 |
| SLS | H | H/Ome | -- | -- | 5-mod dU | 20 | -- | -- | -- | -- | 89 |
| Anti-VWF | ARC1779 | H/Ome | H/Ome | PEG 20k | 3'-3' dT | 1 PS residue | 2,000 | 63; HP | -- | 2; (iv) | 2 | Terminated | 60 |
| ARC15105 | OMe | OMe | PEG 40k | 3'-3' dT | 1 PS residue | 1,000 | >300; RS | >>300; HS | ~67; CM (iv) | -- | -- | 63 |
| Anti-nucleolin | AS1411 | H | H | -- | -- | -- | -- | -- | -- | -- | 2; (iv) | 2 | Terminated | 64,68 |
| Anti-TFPI | BAX499 | H/Ome | OMe | PEG 40k | 3'-3' dT | -- | 3,000 | >>72; HS | -- | 1 | Terminated | 70,72 |
| Anti-hepcidin | NOX-H94 | L-2'OH | L-2'OH | PEG 40k | -- | -- | 650 | >>60; HS | 23 | H (iv) | 2 | Complete | 37,78,79 |
| Anti-CXCL12 | NOX-A12 | L-2'OH | L-2'OH | PEG 40k | -- | -- | 200 | >>60; HS | 40 | H (iv) | 2 | Ongoing | 36,78,79 |
| Anti-CCL2 | NOX-E36 | L-2'OH | L-2'OH | PEG 40k | -- | -- | 500 | >>60; HS | 50 | H (iv) | 2 | Complete | 78,79 |
| Anti-PSMA | ARC591 | F | OH | -- | -- | -- | 1,300–1,800 | ~5; MP | -- | -- | -- | 94 |
| ARC1725 | OMe/F | H/Ome | -- | 3'-3' dT | 1 PS residue | 4,700 | 20; HP | -- | -- | -- | 94 |
| anti-IL-6 | SL1025 | H/Ome | H/Ome | -- | 3'-3' dT | 5-mod dU | 200 | >48; HS | -- | -- | -- | 90 |
| SL1023d | H/Ome | H/Ome | -- | 3'-3' dT | -- | -- | 5.5; HS | -- | -- | -- | 90 |
| SL1026 | H/Ome | H/Ome | PEG 40k | 3'-3' dT | 5-mod dU | 2,500 CM | 0.4, 160; (CM) | -- | -- | -- | 93 |

-- data not available, relevant, or reported.
CM, cynomolgus monkey; H, human; HP, human plasma; HS, human serum; RS, rat serum; i.v., intravenous; s.c., subcutaneous; i.v.t., intratovel; F, 2’fluoro; MU, mouse urine; NH$_2$, 2’ amino; OMe, 2’ O-methyl; H, 2’ deoxy; L-OH, L-2’hydroxy; 3’-3’dT, inverted deoxythymidine; PS, phosphorothioate; PSMA, prostate-specific membrane antigen; PDG, platelet-derived growth factor; VWF, von Willebrand factor; 5-mod dU, deoxyuridine bearing a modification on the 5 position; Approved, clinical trial complete and FDA approved; Terminated, clinical trial terminated; Complete, clinical trial complete; Ongoing, clinical trial ongoing. 4Half-lives ($t_{1/2}$) for clearance are as reported in cited references. $t_{1/2}$ for the $\alpha$ and $\beta$ rates were specified if possible. 4Indicates median duration of effect instead of $t_{1/2}$. 5Stability was assessed on a non-PEGylated variant. 6Denotes insignificant degradation detected at that time point. 7Denotes an IC$_{50}$ for inhibition of NAALDase activity. 8Denotes Levy Lab, unpublished results.

**Additional aptamers approved for phase 1/phase 2 clinical evaluation**

**ARC1779.** Selected to bind the von Willebrand factor (VWF) A1-domain, ARC1179 was originally identified from ssDNA library. The minimized aptamer, composed of 39 nucleotides was subsequently modified systematically to improve stability by replacing 26 of...
the nuclease-labile DNA residues with stable 2’OMe nucleosides, including a single phosphorothioate residue between positions 20 and 21, and by capping at the 3’ end using an inverted dT residue. To increase serum lifetime, the 5’ end of the molecule was synthesized bearing a 5’ amine which was conjugated to a 20kDa PEG moiety. 

ARC1179 displayed high affinity for the VWF A1-domain ($K_D \sim 2$ nmol/l) and specifically inhibited VWF-mediated platelet aggregation ($IC_{50} \sim 300$ nmol/l). In human trials, following bolus i.v. administration, the aptamer displayed a serum half-life of ~2 hours. More importantly, the drug proved safe and well tolerated in phase 1 (ref. 35), and in phase 2 trials was demonstrated to effectively inhibit VWF activity in the treatment of thrombotic thrombocytopenia purpura (ref. 61) as well as cerebral embolism. However, when the compound was delivered s.c. it failed to yield plasma concentrations that could effectively control clinical features of thrombocytopenia purpura relegating treatment regimens to bolus primed continuous infusion.

The limitation on route of administration has most recently been addressed by the identification of a second-generation anti-VWF aptamer, ARC15105, composed of 100% 2’OMe RNA that is conjugated to a larger 40 kDa PEG moiety. Like ARC1179, ARC15105 binds the A1-domain and inhibits VWF-mediated platelet aggregation ($K_D \sim 1$ nmol/l; $IC_{50} \sim 300$ nmol/l). Following both i.v. and s.c. in vivo administration in cynomolgus monkeys, the compound displayed half-lives of ~65 hours in serum. The improved PK values are a likely consequence of both improved stability as well as the use of larger, 40kDa PEG moiety. More impressively, 300 hours following a single 20mg/kg s.c. dose, VWF activity remained >90% inhibited clearly demonstrating the benefit of this dosing method. Future plans for clinical evaluation of ARC15105 are currently unknown.

AS1411. Originally identified during a screen of antisense oligonucleotide for antiproliferative activity, the aptamer AS1411, formerly AGFO001, is a 26 nucleotide DNA molecule composed exclusively of guanosine and thymidine residues that exists in solution as a G-quadruplex. Although the molecule was originally reported to interact with cell surface nucleolin, be taken up by cancer cells, and inhibit cell growth, more recent work has demonstrated a disconnect between cell uptake and nucleolin binding.

In this work, nucleolin-negative cells were shown to display significant (and sometimes greater) levels of AS1411 uptake by cancer cells, and inhibit cell growth, more recent work has demonstrated a disconnect between cell uptake and nucleolin binding. In this work, nucleolin-negative cells were shown to display significant (and sometimes greater) levels of AS1411 uptake by cancer cells, and inhibit cell growth, more recent work has demonstrated a disconnect between cell uptake and nucleolin binding.

AS1411 binding. AS1411 binds the A1-domain and inhibits VWF-mediated platelet aggregation ($K_D \sim 1$ nmol/l; $IC_{50} \sim 300$ nmol/l). Following both i.v. and s.c. in vivo administration in cynomolgus monkeys, the compound displayed half-lives of ~65 hours in serum. The improved PK values are a likely consequence of both improved stability as well as the use of larger, 40kDa PEG moiety. More impressively, 300 hours following a single 20mg/kg s.c. dose, VWF activity remained >90% inhibited clearly demonstrating the benefit of this dosing method. Future plans for clinical evaluation of ARC15105 are currently unknown.

ADVANCEMENTS IN APATMER SELECTION TECHNOLOGY

Mirror image aptamers, Spiegelmers

Genetically, postselection incorporation of backbone modifications results in nonimmmunogenic, nuclease-stabilized nucleic acids, which display increased serum stability allowing for therapeutic applications. However, this “back filling” approach adds time to the R&D process and is limited because not all positions in an aptamer can be changed to more stable analogs. Indeed, all of the aforementioned clinically evaluated aptamers retain at least one 2’H or 2’OH nucleotides, leaving the aptamers more susceptible to serum nuclease degradation.

A very different, yet effective approach for the generation of highly stable molecules is the use of mirror image aptamers, or so called Spiegelmers (Figure 4). Composed of chains of L-nucleotides, as opposed the natural D-nucleotides, and forming left-handed helices instead of the typical right-handed helices, Spiegelmers display exceptional serum stability as they are not recognized by endogenous nucleases and exhibit essentially no degradation in serum. Of course, for this same reason these molecules cannot be made using existing enzymes. As such, Spiegelmers are initially selected using natural D-nucleotides against mirror image targets (i.e., a D-peptide; Figure 4a). The resultant D-aptamers are then synthesized chemically in the inverted stereo configuration as L-aptamers (Spiegelmers), which bind the natural L-peptide or protein target (Figure 4b).

To date, three Spiegelmers have been described with a 40kDa PEG at the S’ end to slow in vivo clearance, which includes the anti-hepcidin lexaptepid pegol (NOX-H94), the anti-CCL2 emapticap pegol (NOX-E36), and the anti-CXCL12 olaptesed pegol (NOX-A12). All three have undergone phase 1 studies where they demonstrated good safety profiles in healthy volunteers. Both NOX-A12 and NOX-H94 have also yielded promising results in phase 2 studies. Additional trials are currently underway with a number of other Spiegelmers in the pipeline (reviewed in ref. 79).

New RNA polymerase variants yield 100% modified RNAs

As noted above, perhaps one of the most significant advancements towards improving the rate of discovering aptamers for clinical evaluation has come from the evolution/creation of novel polymerases that provide a means to incorporate a wider variety of sugar backbone modifications beyond the canonical 2’H and 2’OH. This renders the need for “backfilling”, as was done for pegaptanib,
were described by Meyer et al. By including a series of stabilizing nucleotides, those observed for the wild-type polymerases using unmodified mutations into the enzyme, they were able to further increase recently reported modified RNAs. For example, Friedman ing through the community that are capable of synthesizing 100% naturally occurring protein (a). Once optimized as a D-aptamer the mirror image L-aptamer (Spiegelmer) is synthesized chemically and intrinsically binds to the natural L-target, such as a naturally occurring protein (b).

Figure 4 Mirror image aptamers, Spiegelmers, are composed of non-natural L-ribose nucleotides. The molecules are initially selected from natural D-ribose aptamer libraries against a non-natural target, for example a D-peptide (a). Once optimized as a D-aptamer the mirror image L-aptamer (Spiegelmer) is synthesized chemically and intrinsically binds to the natural L-target, such as a naturally occurring protein (b).

Figure 5 Modified deoxyuridine (dU) residues are at the core of the novel molecules developed by Somalogic. A variety of chemical moieties are attached to the 5-position of dU via a carboxamide linkage (left). A variety of different modifications (R) have been employed for the selection of SOMAmers including benzyl, napthyl, and indole (right).

of utilizing a range of nucleotide triphosphate substrates ranging from 2'F, 2'NH₂, and 2'OMe ribose, to much more exotic cyclohex- enyl, arabinohex-5-enyl, arabin, or-L-threo furanosyl, and 2'-fluoroarabin nucleic acids (Figure 2d). More importantly, his group has used these to demonstrate the generation of so called xeno nucleic acid aptamers. Indeed, xeno nucleic acids may represent the future for aptamers as they have the potential to bring not only added stability and nuclease resistance, but also for novel properties, structures, and folds.

SOMAmers

Although their major focus has been on the development of a diagnostics platform, Somalogic has made significant advancements in the area of utilizing base modifications to give aptamers protein-like functionality. Their slow off-rate modified aptamers (SOMAmers) not only display improved binding affinities and binding kinetics (in particular, slow off-rates) when compared to traditional aptamers, but the inclusion of these modifications in their libraries significantly increased the selection "hit rate".

The linchpin to this functionality arises from replacing the dT residues within ssDNA aptamer libraries with a dU residue chemically modified at the 5-position of the base (Figure 5). With a variety of mostly hydrophobic modifications available at the 5-position, such as benzyl, naphthyl and indole, a number of novel aptamers have been found for previously unselectable targets thanks to these useful modifications.

Thus, it is perhaps amusing that 10 years after the initial DNA selection against platelet-derived growth factor at NexStar, which resulted in Fovista, Janic and colleagues, now at Somalogic, utilized a benzyl-dU modified DNA library to select for anti-platelet-derived growth factor SOMAmers. The resulting SOMAmers were not only backfilled with 2'OMe residues to increase stability, but also subjected to a severe allergic reaction-like study (structure activity relationship) typically used for small molecule compounds to assess how altering the moiety on the modified dU affected function. The resulting molecule, SLS, tolerated 2'OMe stabilization in 4 of the 10 purines with a 20 pmol/l affinity (which the authors admit is approaching the detection limit of the assay), and IC₅₀ for cellular phosphorylation of 50 pmol/l.

The power of this chemical functionality has been further demonstrated through the discovery of a 32 nucleotide SOMAmer, SL1025, which binds IL-6 with 200 pmol/l binding affinity and exhibits very little nuclease degradation over a 48-hour incubation in human serum. Interestingly, the presence of the modified dU moiety was shown to be responsible for a >9-fold increase in the serum stability of these molecules when comparing SL1023 (an all-2'H variant
of SL1025) with SL1023dT (a variant of SL1023 where modified dU was replaced with dT) (Table 1). A subsequent crystal structure led to the realization that a 16 nucleotide domain within the aptamer formed a G-quartet and bound the target with 270 nmol/l binding affinity. The authors then demonstrated the power of the severe allergic reaction-like approach by determining the appropriate combination of benzyl, phenyl, and naphthyl groups within the domain, improving the affinity ~15-fold.

Most recently, studies with the parent SL1025 have now been conducted in non-human primates. Using a rheumatoid arthritis model in cynomolgus monkeys, and SL1026, a variant of SL1025 bearing 40kDa PEG, animals were dosed at 10mg/kg q.i.d. for 11 days. Treated animals displayed a reduction in IL-6 levels and arthritic symptoms over control. Of additional note, when compared to the clearance rate determined from a single injection of SL1026, the sustained dosing schedule resulted in SL1026 serum concentrations 1,000-fold higher than projected on the sixth day of treatment, indicating that the clearance mechanism for the aptamer may have become saturated. However, most importantly for SL1026 and other SOMAmers, despite the presence of the unnatural base modification on the 5 position of the deoxyuridine residues, SL1026 did not induce any detectable immune response in the monkeys. Though there has been no public movement toward trials to date, this work as well as work targeting platelet-derived growth factor leaves little doubt that we will see SOMAmers approved for clinical trials in the not too distant future.

FUTURE AND CONCLUSIONS

The advances described herein have facilitated the production of highly stabilized therapeutic aptamers that have advanced to clinical evaluation. Chemical modifications of the sugar-phosphate backbone, whether made synthetically by solid-phase synthesis, or enzymatically through mutant polymerases, have permitted the generation of aptamers that can withstand in vivo nucleases without inducing immunogenicity. Stereo-aptamers, Spiegelmers, bypass the nuclease and immunogenicity problem by making use of non-natural L-nucleotides. SOMAmers, in addition to incorporating modified ribose sugars, are decorated with moieties that impart chemical function beyond what standard RNA or DNA aptamers could ever achieve. Even though the core selection principle remains the same as the first-generation aptamers, these advances have made possible the rapid production of aptamers with significantly improved function and simultaneously reduced effort in postselection optimization.

It is important to note, the aptamers that have been evaluated in clinical trials thus far have primarily been for binding and inhibition of target function. Extrapolating from the progression of antibodies from use as inhibitors to carriers for the targeted delivery of therapeutics, the utilization of aptamers in a similar capacity is likely not far behind. For example, we and others have performed both laboratory as well as preclinical studies investigating the potential to use aptamers for the targeted delivery of cargoes ranging from small molecule drugs and toxins, to proteins, therapeutic oligonucleotides, and nanoparticles (reviewed in ref. 5). Indeed, further exploration of the patent literature reveals work from Archemix that was focused on developing optimized, minimized, and stabilized variants of the aptamer A9 for clinical delivery. A9, an aptamer originally reported by Lupold et al., targets the prostate-specific membrane antigen, a protein not just associated with prostate cancer, but found on the neovascularature of most solid tumors. Starting with a 2’F modified aptamer, the minimized molecule, ARC591, was back filled and stabilized using a combination of 2’OMe, 2’H, and a phosphorothioate linkage to yield a molecule (ARC1725) with a 20-hour degradation half-life in human serum, making the aptamer primed for eventual clinical testing (Table 1). We anticipate that it will not be long before aptamers engineered for delivery of therapeutics debut in clinical trials against prostate-specific membrane antigen and other targets.

With many advances in place, several aptamers in clinical trials, and others in the pipeline, the future of therapeutic aptamers looks extremely hopeful. Where the field progresses from here is uncharted; however, it seems clear the next generation of therapeutic aptamers will be generated more rapidly, encompass more functionality, and require less postselection stabilization than those that have or are currently undergoing clinical testing. Building from the existing advances, it would seem that these aptamers will combine enhanced functionality, as demonstrated by SOMAmers, with polymerases that can incorporate nuclease stabilizing ribose modifications, into the selection. Those advancements, considered with the modern ease of synthesizing oligonucleotides in vast numbers and in large scale, leaves aptamers poised to move toward clinical evaluation and eventual US Food and Drug Administration approval even more quickly.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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