Discovery and Development of Therapeutic Aptamers

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Key Words

SELEX, metabolism, pharmacokinetics, toxicology, PEG

Abstract

Therapeutic aptamers are single-stranded structured oligonucleotides that bind to protein targets with high affinity and specificity and modulate protein function. Aptamers are discovered by iterative rounds of selection for binding to the target protein, partitioning, and amplification of binding clones from a diverse starting library (SELEX). Postselection optimization of clones using chemical modification is directed at improving affinity, potency, and metabolic stability. A key attribute of therapeutic aptamers is the ability to tailor the pharmacokinetic profile by modulating the degree of metabolic stability and modulating renal clearance and rate of distribution by conjugation to various sizes of polyethylene glycol (PEG). In toxicology studies, therapeutic aptamers have been largely devoid of the previously reported oligonucleotide class effects of immune stimulation, complement activation, and anticoagulation; and the principal finding is the histologically visible accumulation of drug-related material in mononuclear phagocytes, a finding generally not considered an adverse effect. Good safety margins between the pharmacologically effective dose and toxicologically established no-adverse-effect levels have been observed consistently. There are presently seven aptamers either on the market or in clinical trials, but there is still much to be demonstrated in terms of chronic systemic use to fully realize the potential of this promising new class of drugs.

INTRODUCTION

SELEX: systematic evolutions of ligands by exponential enrichment

Aptamers are single-stranded oligonucleotides that form stable three-dimensional structures capable of binding with high affinity and specificity to a variety of molecular targets. Aptamers bind to protein targets in much the same manner as antibodies and modulate protein function. To one unfamiliar with aptamers, it may at first seem unintuitive that oligonucleotides could act in such a manner; but on brief reflection, high-affinity and high-specificity interactions between oligonucleotides and proteins occur naturally in many biological processes including transcription, translation, RNA interference, and others. Once the concept of high-affinity oligonucleotide-protein interaction and its functional consequence is accepted, one can imagine that this potential can be exploited pharmacologically to discover nonnaturally occurring oligonucleotides that bind to target proteins of interest and modulate protein function. This is the theoretical basis for therapeutic aptamers, and this therapeutic principle has been developed to the point where there is now one marketed product [Macugen^{TM}, an anti-vascular endothelial growth factor (VEGF) aptamer] and several aptamers in clinical development today.

Several reviews have been written that overview the aptamer discovery process, general pharmacological properties, and even the clinical pharmacology of several aptamers in development today (1–10). In this paper, we briefly review how aptamers are discovered, with a focus on recent advances that improve the pharmaceutical properties of aptamers as a class. We then focus on aspects of the technology that have not been reviewed previously, including the drug metabolism (DM) and pharmacokinetics and the toxicology of aptamers as a class. Finally, we review the status of the development of aptamers that have progressed into clinical trials or to the marketplace and conclude with a discussion about strengths and weaknesses of the technology as it exists today and where we believe opportunities lie for improving and expanding the use of this therapeutic class.

THERAPEUTIC APTAMER DISCOVERY

Aptamers are discovered using a selection-based process known as systematic evolution of ligands by exponential enrichment (SELEX). Tuerk & Gold first described the SELEX process in 1990, and variants on the process have been described many times since then (8, 11, 12). The essential elements of the SELEX process are illustrated in **Figure 1** and briefly described here.

As a starting point, the SELEX process relies upon a large library of single-stranded oligonucleotide templates derived from a chemical synthesis on a standard DNA synthesizer. Template molecules typically contain fixed 5′- and 3′-terminal sequences for molecular biology manipulation that flank an internal region of 30–40 random nucleotides. The starting library, generally $\sim 10^{14}$ unique sequences, is allowed to come to equilibrium with the target protein. A fundamental component of the SELEX process is the partitioning step, in which members of the library that bind to the target are separated from those that do not, using one of several possible partitioning methods. The bound clones are eluted and amplified, thus enriching the pool with sequences that bind to the target protein. Because a single round of selection is not entirely efficient at recovering only the small number of high-affinity binding sequences from the pool, the selection process is repeated iteratively through rounds of progressive target-pool incubation, partitioning, and amplification resulting in the recovery of aptamers that have high affinity and specificity toward the target. The entire SELEX process is generally completed in ~7 to 15 rounds of selection, with an end result of the identification of a small number (~1–100) of full-length binding clones that represent hits for further post-SELEX processing and optimization.

Variations on the SELEX process include different random region lengths and partitioning methods, but perhaps more importantly, different chemical modifications of the nucleosides or

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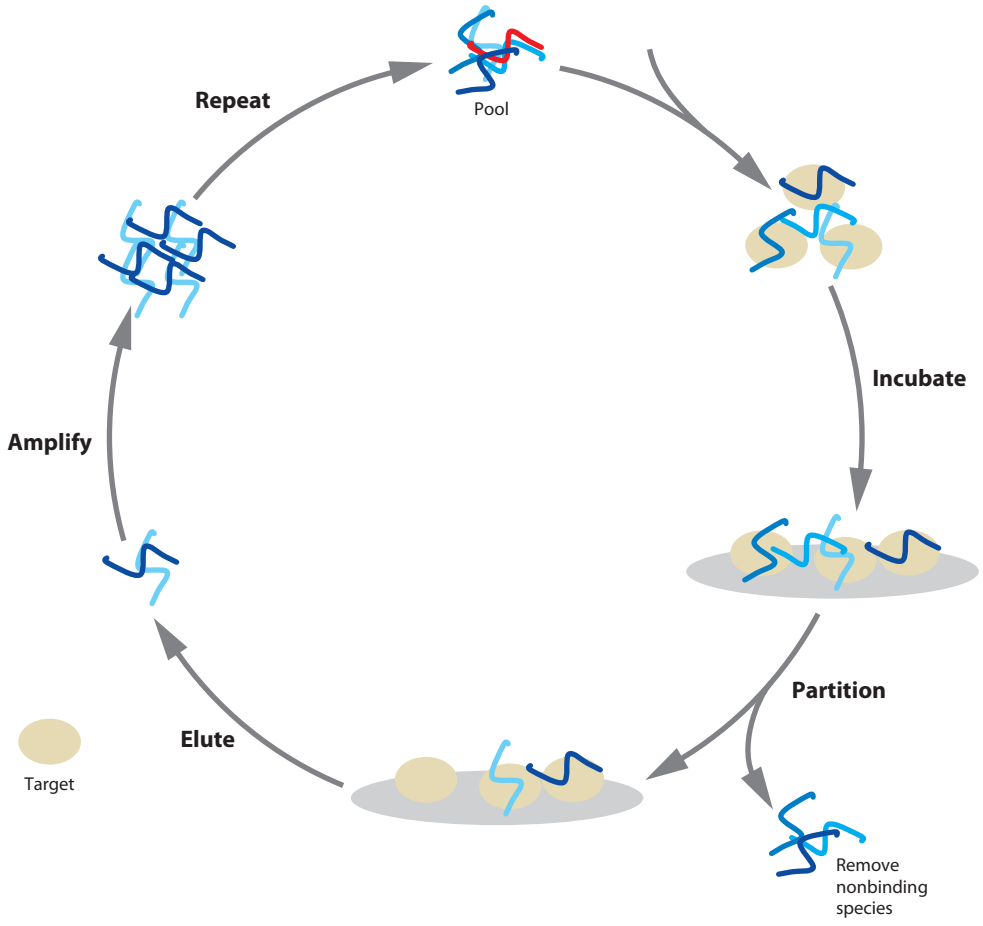

Figure 1

The SELEX process.

nucleotides in the starting pool. The composition of the starting pool can be unmodified DNA or RNA, but oligonucleotides composed of these naturally occurring nucleic acids are readily degraded by nucleases and are therefore suboptimal for most therapeutic applications because of their metabolic instability in biological matrices. Various chemical modifications to impart nuclease stability to oligonucleotides have been studied in the context of aptamers and other therapeutic oligonucleotide classes. Stabilizing modifications can be incorporated into starting pools or incorporated into the aptamers post-SELEX (see optimization process below). For incorporation into SELEX pools, it is important that the modifications are also compatible with the molecular biology steps required to execute the SELEX process. Modifications that have been incorporated successfully into starting pools include replacement of the 2′H (DNA) or 2′OH (RNA) position of the ribose ring of pyrimidines with a 2′F or 2′OMe group utilizing mutated forms of RNA polymerase that can incorporate these modified nucleotides into the selection library (13). Recently, an RNA polymerase variant was developed at Archemix Corp. that can synthesize fully 2′OMe-modified RNA transcripts (unpublished results). These modifications impart substantial nuclease resistance to the resultant oligonucleotides. An interesting alternative approach

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PEG: polyethylene glycol

PEGylated: chemically conjugated to a polyethylene glycol molecule

DMPK: the study of drug metabolism and pharmacokinetics

to metabolic stability is Spiegelmer technology (spiegel is the German word for mirror), in which SELEX using RNA pools is performed against the unnatural, mirror-image configuration (i.e., the D-enantiomer) of a target peptide (14). The identified RNA sequence can then be synthesized in the opposite chirality using mirror-image L-nucleotides to yield a Spiegelmer that binds to the natural form of the target. This process has been demonstrated repeatedly to be an effective way to discover nuclease-resistant aptamers against a number of different peptide targets (14–19).

Once full-length clones with appropriate binding and/or functional characteristics are identified, they are optimized for better drug-like properties. The clone is truncated or minimized to the smallest binding entity that maintains the desired binding affinity for the target. Minimizing the aptamer to the smallest possible size is important for downstream ease of solid-phase synthesis and to limit costs associated with manufacture of the resultant aptamer. Fully minimized aptamers are generally 15–45 oligonucleotides in length and have a molecular weight of ~5–15 kDa. Modifications are then introduced systematically through chemical synthesis to impart improved metabolic stability or enhancement of binding affinity. These may include alternative substitutions (e.g., 2′OMe, 2′F) at the 2′ position of the sugar, substitutions altering the phosphate backbone [e.g., introduction of sulfur in place of nonbridging phosphodiester oxygen (P=S)], and nucleoside substitutions. Chemically modified aptamers are then assayed for the desired activity. This analysis creates a structure-activity map of the molecule that identifies positions at which a given substitution increases, has no impact on, or decreases binding affinity and/or functional activity. The use of fully stabilized (e.g., all 2′OMe) RNA pools at the outset of SELEX may reduce the need for subsequent profiling studies aimed at improving metabolic stability. Beneficial or tolerated substitutions identified in scans can be combined to build composite molecules whose binding and stability properties are fully optimized. Typically, an optimized lead molecule has numerous and diverse chemical modifications.

Depending on the intended use of the aptamer and desired in vivo characteristics, an optimized lead molecule with appropriate binding affinity and metabolic stability may be conjugated at one (5′) or both (5′ and 3′) ends to a large-molecular-weight moiety such as polyethylene glycol (PEG). Conjugation to PEG increases the molecular mass of the molecule and extends its elimination half-life by slowing renal filtration and distribution from the central compartment. PEG conjugation has been used extensively with proteins, peptides, small molecules, and oligonucleotides as a strategy to extend the circulating half-life (20–24). Because aptamers are synthesized by solid-phase chemical synthesis, conjugation chemistry is possible at any position in the molecule. This allows for site-specific functionalization and conjugation of the molecule to a wide variety of chemical moieties, including other oligonucleotides (siRNAs or another aptamer), to the surface of a nanoparticle or to a small molecule as a vehicle for targeted delivery.

The fully optimized (and usually PEGylated) lead molecule is then ready for further in vitro and in vivo profiling of its pharmacological, DMPK (drug metabolism and pharmacokinetics), and toxicological properties. We do not attempt to review the many specific examples of the pharmacological properties of aptamers here, as they are entirely specific to the target biology, and there have been dozens of published examples (refer to References 1–10). However, to generalize, most aptamers described to date have a common mechanism of action whereby they bind to a specific target protein with high affinity and thereby block the interaction of that protein with another endogenous protein and disrupt a protein-protein interaction as a competitive inhibitor. From a mechanism of action point of view, aptamers therefore function similarly to blocking antibodies or peptides. They tend to have very simple and predictable concentration-effect relationships in vitro and in vivo, as would be expected with competitive ligand binding. One simple illustration can be observed with the anti–von Willebrand Factor (vWF) aptamer ARC1779 tested in vivo in cynomolgus monkeys (**Figure 2**) (25). In this example, the relationship between plasma

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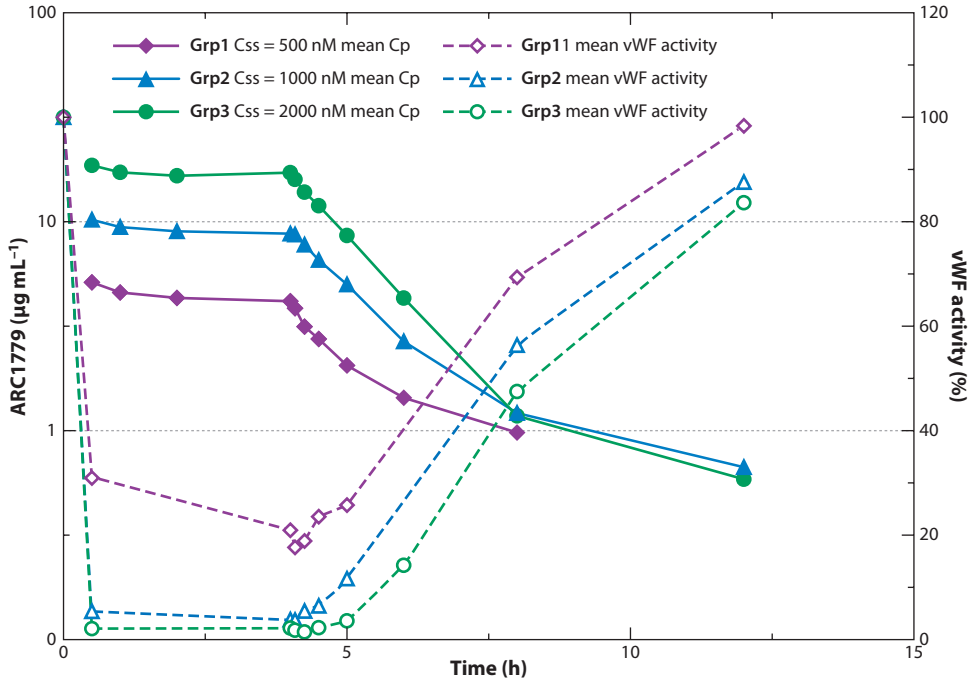

Figure 2

Relationship between plasma concentration and vWF activity in monkeys administered ARC1779 as an IV bolus followed by 4-hour infusion at doses intended to maintain ARC1779 plasma concentrations of 500, 1000, and 2000 nM.

concentrations of ARC1779 and "vWF activity" was measured using an ELISA format where the aptamer competitively inhibits an anti-vWF antibody from binding to an overlapping epitope on vWF (the A1 domain). In this example, with a readily monitored blood-based measure of activity (pharmacodynamics), one can see the simple reciprocal concentration-activity relationship.

THERAPEUTIC APTAMER METABOLISM AND PHARMACOKINETICS

When unmodified oligonucleotides were first studied as potential therapeutics, three major DMPK hurdles were encountered following systemic administration: metabolic instability, rapid renal filtration and elimination of non–protein-bound oligonucleotides, and rapid biodistribution from the plasma compartment into the tissues (26). These properties are generally true for all therapeutic oligonucleotide classes, but the specific challenges they impose vary depending on the class. For aptamers, most targets are soluble or cell-surface proteins present in the blood or interstitial fluid compartments. For most aptamer therapeutics, it is desirable to have the aptamer remain in the plasma compartment for an extended period of time. To accomplish this, aptamers must be modified to increase metabolic stability, slow distribution from the central compartment, and slow renal filtration and elimination.

Nucleic acids composed of naturally occurring nucleotides (RNA and DNA) are not normally found outside of cells to an appreciable extent, and when present outside of the cell they are rapidly degraded by nucleases, and the constituent nucleosides are further metabolized through the endogenous purine and pyrimidine metabolism pathways (27). Therapeutic oligonucleotides

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Table 1 Bioanalytical Techniques for Aptamers and PEGylated Aptamers

Parameter	Bioanalytical Method	Approximate LLOQ (ng/mL)	Detectable Analytes	Specificity
Charge	CGE-UV	1000	Oligo and metabolites	++++
Charge	SAX (IEX)-HPLC-UV	100	Oligo, PEGoligo, and metabolites	++++
Mass	RP-ESI-LC-MS	10	Oligo and metabolites	++++
Mass	MALDI-TOF-MS	10	Oligo and PEGoligo	++++
Binding	Oligreen®	520	Oligo, PEGoligo, and metabolites	+
Binding	Hybidization ELISA	1	Oligo, PEGoligo, and metabolites	++
Radiolabeled	Liquid Scintillation Counts	<0.1	Oligo, PEGoligo, and metabolites	++
Radiolabeled	Autoradiography	<0.1	Oligo, PEGoligo, and metabolites	++

LLOQ, lower limit of quantification.

are metabolized by these same endogenous nucleases. Unmodified RNA and DNA therapeutics are rapidly degraded in the blood and tissues, and have only limited applications owing to the short duration of action. As described earlier, several chemical modifications have been described and are routinely utilized to impart nuclease resistance and resultant metabolic stability. For aptamers, the most frequently used modifications are 2′ OMe or F substitutions and P=S modification.

The bioanalytical toolbox for aptamers is complex and evolving. Several methods can be applied to qualitatively and quantitatively detect aptamers and their metabolites in various biological matrices (**Table 1**). Some of these methods are common to other classes of therapeutic oligonucleotides, but because of the routine conjugation to PEG, additional or alternative methods and approaches are required. No single bioanalytical method is adequate to fully study a PEGylated aptamer, and it is standard practice to use multiple methods orthogonally to achieve the dual goals of high analytical sensitivity and specificity. As shown in **Figure 3**, two different assays, SAX (strong anion-exchange) HPLC (high performance liquid chromatography) and a hybridization assay, were used to determine the concentration of 40-kDa PEGylated aptamer in plasma, vitreous, aqueous, and pooled retina obtained from Dutch-belted rabbits that were dosed with a 40-kDa PEGylated aptamer via single intravitreal (IVT) administration.

Metabolic optimization is an essential part of the lead optimization process. Depending on the intended clinical use and the desired pharmacokinetic profile, lead criteria for metabolic stability can be defined, and the optimization process will seek to generate molecules with very specific characteristics. In most instances, chronic systemic administration is the intended use, and the goal of optimization is to produce the most metabolically stable molecule possible. The chemistries that are routinely employed and the manner in which they are systematically incorporated during lead optimization are described above. As substituted derivatives with the desired target-binding affinity and biological function are produced, a subset of those molecules are tested for metabolic stability and metabolite profiling. In most instances, the relevant matrix for stability assessment is serum. Initially the pre-PEGylated core aptamer is incubated in serum for up to 72 hours at 37°C, and the rate of disappearance of parent molecule is determined. This can be assessed qualitatively in a high-throughput manner by running ^{32}P- labeled aptamer on a polyacrylamide gel electrophoresis (PAGE) or quantitatively using SAX HPLC. During lead optimization, metabolic stability is routinely assessed initially in rat and human serum, and sometimes additionally in mouse, cynomolgus monkey, or other relevant test species. We routinely observe that rodent serum is metabolically more active than human and monkey sera and that this difference translates to more rapid clearance and shorter $t_{1/2}$ in the rat (or mouse) compared with monkey and human. Typically, polyacrylamide gel electrophoresis is used as a high-throughput method during early

SAX: strong anion exchange

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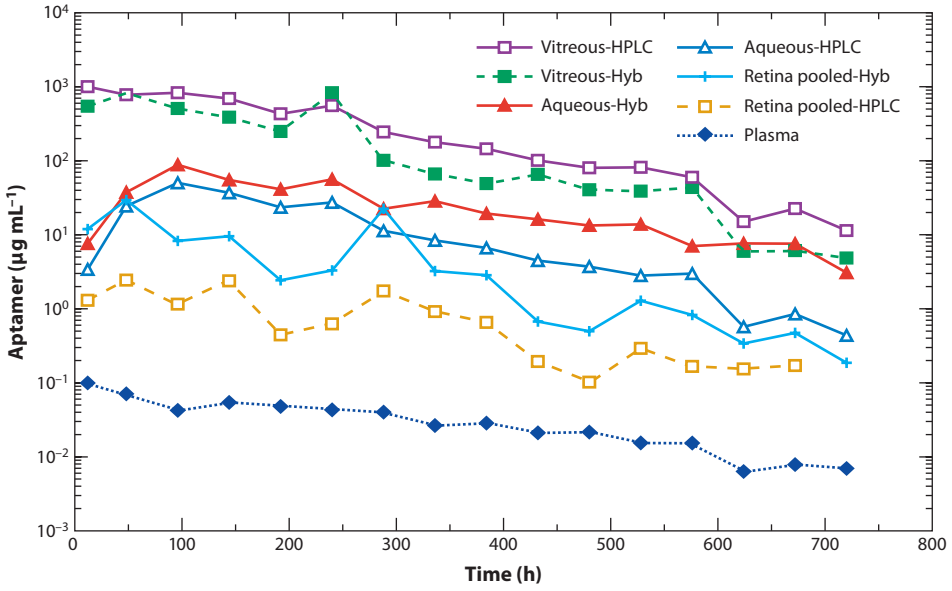

Figure 3

Concentration-time profiles of a 40-kDa PEGylated aptamer as determined by SAX HPLC and hybridization assays in plasma, vitreous, aqueous, and pooled retina of Dutch-belted rabbit after a single 3-mg IVT administration.

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optimization to crudely assess the effectiveness of the optimization, and then SAX HPLC is used late in optimization to establish a quantitative assessment and establish if the late-lead molecules meet lead criteria for metabolic stability.

When optimization has been narrowed to a single series of related molecules, a prototype from the series can be profiled in detail for metabolite identification. This is accomplished by incubation in rat or human serum and by determining the putative identity of the metabolites by molecular mass using liquid chromatography with tandem mass spectroscopy (LC/MS/MS) (28). These experiments generate a tremendous amount of data, as there are typically many primary and secondary metabolites in the sample. It is both a bioinformatics challenge to present all the data and an interpretive challenge to examine the data and impute the principle metabolic hot spots; but once these data are available, subsequent optimization can be site-directed rather than random, allowing for a more focused approach to metabolic optimization. Whereas the specifics of the methods we have described are unique to aptamer therapeutics, the principles are familiar and analogous to the process of metabolic optimization for small molecules.

Once the optimization is completed and a lead is identified, one enters the phase of full characterization of the absorption, distribution, metabolism and elimination (ADME) properties of the lead molecule. The overall objectives of an ADME program are no different than for any therapeutic candidate in terms of the key questions to be answered and the timing of studies relative to the overall development program (**Table 2**). However, there are a number of unique considerations for oligonucleotides and aptamers in the design and interpretation of the program. First, as outlined above, several bioanalytical methods are required throughout the program, each bringing unique strengths and weaknesses. Second, whereas it is clear that nucleases are principally responsible for the metabolic instability of oligonucleotides, nucleases are poorly understood as drug-metabolizing enzymes. Questions such as interspecies differences, induction and suppression, and

LC/MS/MS: liquid chromatography with tandem mass spectroscopy

Table 2 Typical DMPK Studies During Phases 0 to 3 of Clinical Development

Bioanalytical Support	Phase of Development
Development and validation of HPLC assays for rat, monkey, and human plasma (other species as necessary)	Pre IND
Development and validation of immunogenicity assay	Pre IND
Development and validation of pharmacodynamic activity assay	Pre IND
Reference standard synthesis of parent compound	Pre IND
Reference standard synthesis for key impurities or potential metabolites	Pre IND
Development and validation of more sensitive assays in humans (hybridization or LC/MS/MS)	Phase 2/3
Synthesis of major metabolites	Phase 2/3
PK/ADME	
PK profile in mouse, IV	Pre IND
PK profile and dose proportionality in rat, IV	Pre IND
PK profile and dose proportionality in monkey, IV	Pre IND
Rat PK profile and dose proportionality by the selected clinical route of administration	Pre IND
Monkey PK profile and dose proportionality by the selected clinical route of administration	Pre IND
Radiolabeled $^{14}C/^{3}H$ rat mass balance and in vivo metabolic profiling in urine and feces after single IV dose	Pre IND-Phase 2
Radiolabeled $^{14}C/^{3}H$ rat tissue distribution (QWBA) and tissue metabolic profiling after single IV dose	Pre IND-Phase 2
Radiolabeled $^{14}C/^{3}H$ monkey mass balance and in vivo metabolic profiling in urine and feces after single IV dose	Phase 2/3
Radiolabeled $^{14}C/^{3}H$ monkey tissue distribution (QWBA) and tissue metabolic profiling after single IV dose	Phase 2/3
Radiolabeled $^{14}C/^{3}H$ rat lacteal secretion and placental transfer mass balance	Phase 2/3
Radiolabeled $^{14}C/^{3}H$ rat placental transfer by quantitative tissue distribution	Phase 2/3
Radiolabeled $^{14}C/^{3}H$ rat placental transfer by quantitative whole body autoradiography	Phase 2/3
Radiolabeled $^{14}C/^{3}H$ rat mass balance and in vivo metabolic profiling in urine and feces after repeated dosing	Phase 2/3
Radiolabeled $^{14}C/^{3}H$ rat tissue distribution (QWBA) and tissue metabolic profiling after repeated dosing	Phase 2/3
Radiolabeled $^{14}C/^{3}H$ monkey mass balance and in vivo metabolic profiling in urine and feces after repeated dosing	Phase 2/3
Radiolabeled $^{14}C/^{3}H$ monkey tissue distribution (QWBA) and tissue metabolic profiling after repeated dosing	Phase 2/3
Radiolabeled $^{14}C/^{3}H$ rat single dose biliary excretion	Phase 2/3
Special PK/ADME studies to resolve specific issues as PK/ADME data generated	Phase 2/3
In Vitro Metabolism	
Interspecies metabolism and metabolic profiling in rat, monkey, and human serum	Pre IND
Interspecies metabolism and metabolic profiling in rat, monkey, and human S9 fraction or cytosol	Pre IND
Drug-drug interaction; inhibition kinetic studies	Pre IND
In vitro rat, monkey, and human plasma protein binding with core aptamer and PEGylated aptamer	Pre IND
Other Studies	
Allometric scaling using single-dose mouse, rat, and monkey (+/− others) PK profiles	Pre IND
PK/PD in animal model	Pre IND
Renal or other organ of elimination compromised study(ies)	Pre IND-Phase 3

saturation, which are routinely studied and understood elements of small-molecule metabolism, are still unknown for nucleases. Third, the choice of the most relevant test species is generally based on comparative pharmacology and historical precedence with oligonucleotides, rather than knowledge of comparative pharmacokinetics (PK) and metabolism, as is commonly used in species selection for small molecules. Fourth, conjugation with large-molecular-weight PEG introduces a number of technical challenges and considerations that are common to any PEGylated product. PEG is a polydispersed hydrophilic molecule that dominates the physical properties of the

PK: pharmacokinetics

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final conjugated molecule and challenges the specificity of bioanalytical methods. Fifth, oligonucleotides are macromolecules and have long been considered at least potentially immunogenic. Whereas the evidence to date with all oligonucleotide therapeutics including aptamers indicates that they are not immunogenic, this remains a question that needs to be continuously studied with relevant biological assays. Lastly, the study of in vivo metabolism and fate is a complex endeavor, especially with PEGylated molecules, and it is not clear at present how extensively these topics should be investigated for this class of molecules.

ASO: antisense oligonucleotide

QWBA: quantitative whole-body autoradiogaraphy

All aptamer therapeutics described to date have been administered by intravenous (IV), subcutaneous (SC), or intravitreal (IVT) administration. Whereas other routes (inhalation, mucosal, dermal, intrathecal) would appear to be technically feasible based on earlier work with other oligonucleotides, they have not yet been studied for aptamers, and the discussion of absorption here is restricted to IV, SC, and IVT. By definition, aptamers are fully bioavailable following IV administration; however, the determination of plasma total exposure may be limited by the sensitivity of the bioanalytical method. It is typical for early SAX HPLC-based bioanalytical methods to have sensitivities in the 100–500 ng/mL range. As a result, the β-elimination phase may be only partially characterized, and the low-amplitude γ-phase is likely to be missed entirely. The same holds true for bioanalysis following SC or IVT administration, but a larger proportion of the total exposure may be missed, and the determination of absolute bioavailability (F) may be influenced by this factor; thus, it is important to develop sufficiently sensitive methods as early as possible during development.

The PK following IVT administration has been determined for the PEGylated stabilized anti-VEGF aptamer Macugen™ and a similar composition anti-TGF-B aptamer, and in both instances the F was high (29, 30). Following SC administration, we have studied several PEGylated and partially or fully stabilized aptamers in the rat and cynomolgus monkey. Subcutaneous absorption is slow, with T_{max} typically occurring in 24–48 hours, and bioavailability is generally low in the rat and variable but often quite high (e.g., >80%) in the monkey. Although there is still a limited data set, it appears that there is a positive correlation between the degree of metabolic stability and the observed subcutaneous F, as has been shown with antisense oligonucleotides (ASOs) (31). A typical IV-SC cross-over PK study in the cynomolgus monkey is shown in **Figure 4**, demonstrating PK that would be favorable for chronic intermittent SC administration and with an overall PK profile similar to a therapeutic antibody.

Biodistribution of oligonucleotides can be studied quantitatively using radiolabeled compounds followed by quantitative whole-body autoradiography (QWBA) or using organ removal and scintillation counting. On a more limited basis, analytical quantification can be performed following tissue extraction with un-PEGylated compounds with substantial investment in tissue extraction methods. Quantitative methods can be complemented by qualitative methods such as immunohistochemistry (when an antibody against some element of the therapeutic oligonucleotide exists), in situ hybridization, or microautoradiography. These latter methods provide a better appreciation for the microanatomic distribution and the specific cell types associated with the presence of material. In addition, because high concentrations of oligonucleotides can be visualized histologically within tissues from toxicology studies, the quantitative biodistribution data are further complemented by observational data from toxicology studies (32).

At present there are limited data on the biodistribution of therapeutic aptamers, but there are appreciable published data on the biodistribution of ASOs with which available aptamer data can be compared. Distribution of ASOs from the plasma compartment to the tissues is rapid and broad, but with the highest concentrations typically observed in the liver, kidney, spleen, lymph nodes, and bone marrow (33). In these tissues, the majority of the compound is present in cells of the mononuclear phagocyte system such as Kupffer cells of the liver, macrophages in the

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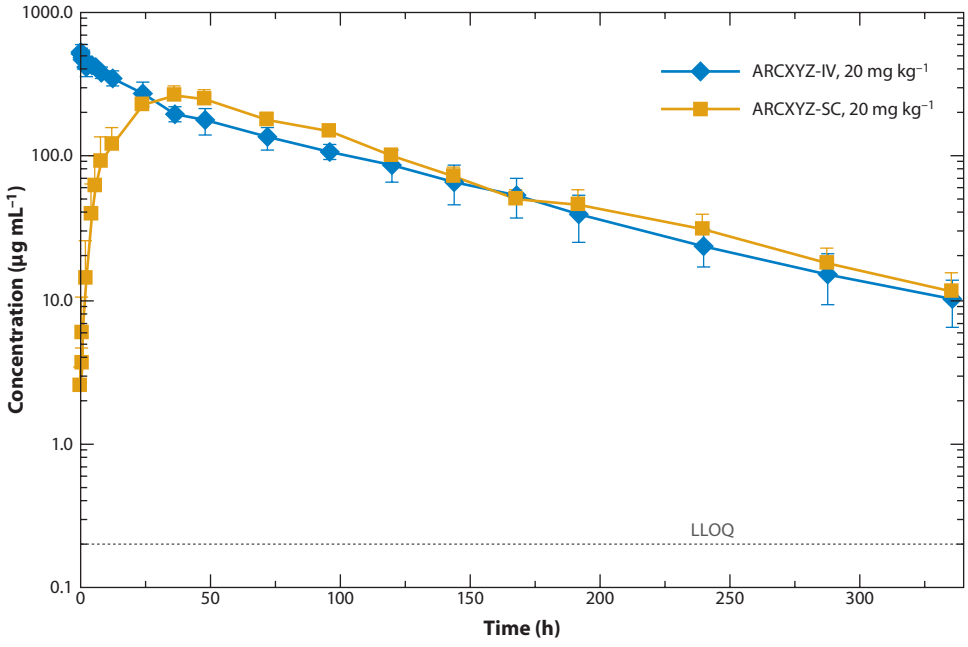

Figure 4

Concentration-time profile of a fully stabilized 40-kDa PEG-conjugated aptamer following IV or SC bolus administration to cynomolgus monkeys at 20 mg/kg.

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spleen, and sinusoidal macrophages of lymph nodes, or in the kidney, associated with the reuptake of filtered oligonucleotides into the proximal tubular epithelium. Quantitative biodistribution data with therapeutic aptamers are limited to data on Macugen™ (34), an exploratory study involving pharmacologically inactive test aptamers (35), and our unpublished data with the anti-vWF aptamer ARC1779. In all cases, these are PEGylated and partially stabilized aptamers.

Representative images from a QWBA study of ARC1779 show several important observations (**Figure 5**). Radiolabeled compound is initially seen mainly in the blood, followed by distribution most notably to the liver, spleen, lymph nodes, bone marrow, and kidneys (and urine). This pattern is entirely consistent with that generally reported for ASOs. By comparative analysis with published data for ASOs, the addition of the PEG slows the rate of exit from the blood to tissues and decreases the rate of filtration by the kidney, but otherwise the general biodistribution pattern of PEGylated aptamers appears comparable to ASOs. Once distributed to the tissues, radiolabeled material is slowly cleared over time, with ~50% of the material still in the body 2 weeks after dose administration in this example. The slow clearance of stabilized oligonucleotide from tissues is also consistent with data reported with ASOs where tissue half-lives of 30+ days are typical (36–38).

The principles that should be applied to the study of aptamer metabolism are the same as with any drug. To the extent that metabolic fate can be reasonably studied, it should be thoroughly understood. The initial aspects of in vitro metabolism were described above in the discussion of metabolic optimization. The same analytical methods of SAX HPLC, LC/MS/MS (un-PEGylated aptamers only), and radiolabel are used to study comparative in vitro and in vivo metabolism and establish the major and minor metabolites and comparative metabolism across species. The specificity of the assays is critical, and their ability to distinguish parent molecules from metabolites

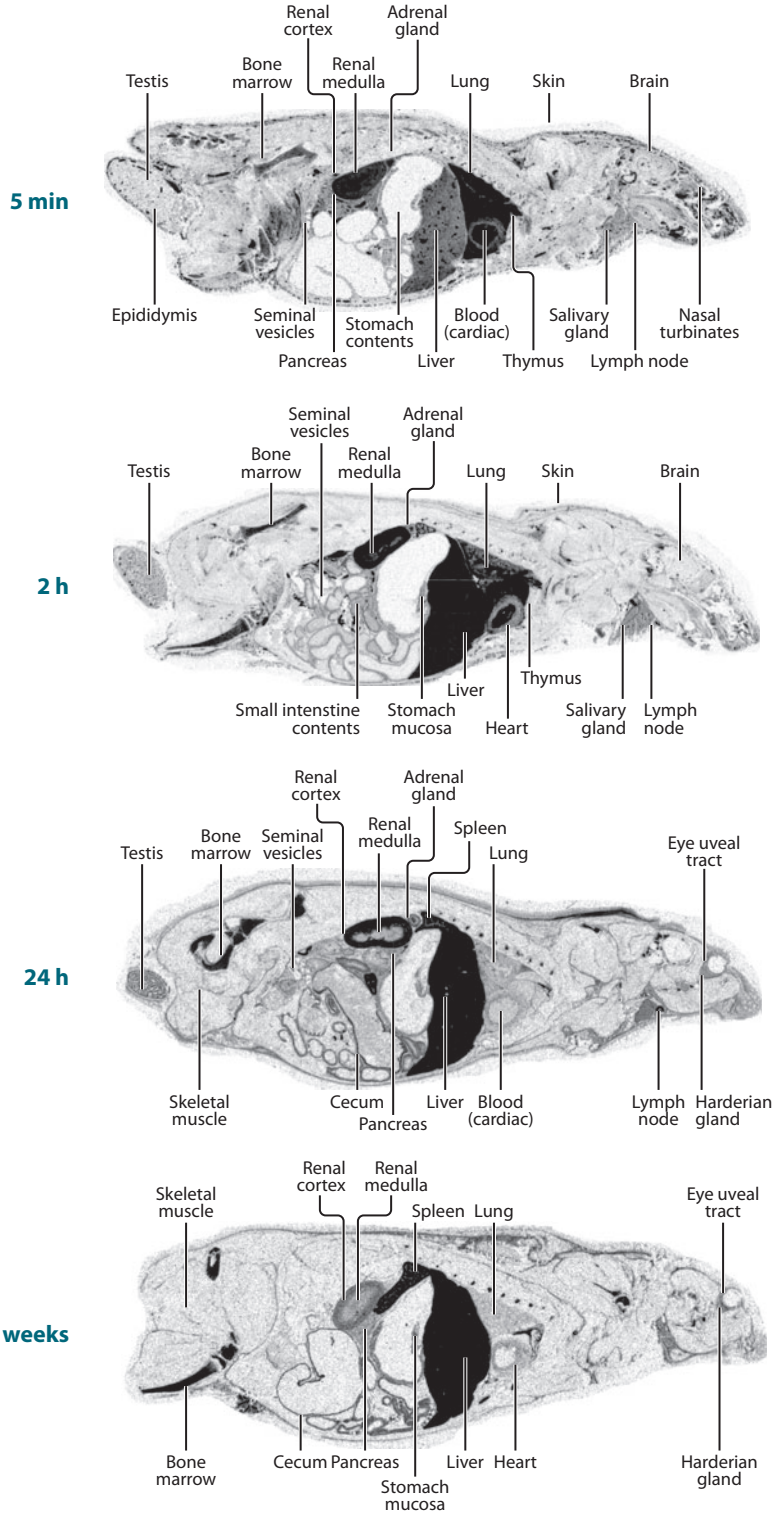

Figure 5

Whole body autoradiograms at 0.083 and 336 hours after single IV administration of [^{14}C]-ARC1779 at a target dose of 16 mg/kg in rat.

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should be established by qualification and validation of the assays using authentic standards of known and putative metabolites. We have observed that aptamers are extensively and progressively metabolized both in vitro and in vivo, and the pattern of degradation indicates that both exonucleases (from the 5′ and 3′ ends) and endonucleases (internal internucleotide bonds) can contribute to aptamer metabolism. The overall metabolic lability and pattern of metabolism is strongly influenced by the stabilizing chemical modifications incorporated into any given molecule.

CYP450: cytochrome P450 enzymes

The potential for metabolism-based drug-drug interactions should be addressed. One would not expect that aptamers will be substrates for cytochrome 450 (CYP450) or phase 2 enzymes, and to date we have not observed evidence of this. Additionally, we have not observed that aptamers induce or suppress CYP450 enzymes. We have not seen evidence for induction or suppression of aptamer metabolism with repeated dose administration, nor have we seen evidence of saturation of metabolism at any doses or plasma concentrations in nonclinical studies.

The elimination of aptamers is best studied using radiolabeled material in mass balance studies. There are only very limited quantitative data on elimination of aptamers to date, but existing data are consistent with other oligonucleotides showing that elimination is almost entirely by renal elimination of intact parent molecule or metabolites. The rate of elimination is influenced by factors such as PEGylation, which slows renal filtration; serum protein binding, which reduces the free fraction and slows filtration; metabolic stabilization, which slows the rate of metabolism in the blood and tissues; and the rate of breakdown and ultimate release of degradation products from intracellular tissue stores. In the example shown in **Figure 5** above (QWBA images), the rate of elimination in the rat is slow, with only ∼50% of the administered material eliminated over a 2-week period and the rest remaining in the carcass. Based on observations of the persistence of visible test-article-related material in tissues from toxicology studies, almost irrespective of chemical composition or PEGylation, we believe this reflects a generalizable pattern for stabilized and PEGylated aptamers characterized by extensive distribution to tissues, uptake by mononuclear phagocytes, slow degradation within intracellular storage compartments, escape of small degradation products from cells into the blood, and then elimination of degradation products through the kidney into the urine.

With these general ADME properties, one can consider what constitutes a desirable plasma PK profile for any given aptamer therapeutic and attempt to design the aptamer to meet that target product profile. Because most therapeutic aptamer targets are extracellular or cell-surface proteins, the plasma is the relevant compartment to study. As one can readily surmise, based on the discussion of ADME properties, a unique strength of aptamers as a therapeutic class is the ability to tune the plasma PK profile by modulating the degree of metabolic stability, the presence or absence of PEG, and the molecular weight of the PEG conjugated to the aptamer. For example, a short-acting antithrombin aptamer was designed to have a very short plasma $t_{1/2}$, and thus a short duration of action, to be used as a continuous infusion during cardiothoracic surgery. This molecule, ARC183, is a 15-mer, all-DNA, un-PEGylated aptamer with a plasma half-life of ∼10 min that correlates with a rapid return of normal coagulation upon cessation of the infusion without the need for a reversal agent (39). An intermediate-duration half-life molecule is represented by the anti-vWF aptamer ARC1779, an antiplatelet therapeutic designed for use in acute care settings (25). This aptamer is a 40-mer, mixed-DNA, 2′OMe modified composition with a 20 kDa 5′ PEG conjugate. ARC1779 has an elimination half-life of ∼2–4 h and can be given as a single IV slow bolus or a continuous infusion (40). Lastly is an example shown above of a long-acting aptamer intended for chronic intermittent SC administration (**Figure 4**). This aptamer is all 2′ OMe modified and has a 5′ 40 kDa PEG conjugate that has an elimination $t_{1/2}$ of ∼75 h and 100% F_{SC} in the cynomolgus monkey. These three examples illustrate the tunability of therapeutic aptamer PK properties by rationale design.

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THERAPEUTIC APTAMER TOXICOLOGY

There is very limited information on the toxicological properties of aptamers except for publically available government and regulatory agency approval reviews for Macugen™ (34). The testing program conducted for Macugen™ represents a limited toxicological assessment because of the low total dose administered (0.3 mg/eye), intermittent administration (every 6 weeks), compartmental administration (IVT), and the resultant minimal systemic exposure. As a consequence, whereas there is ample publically available toxicology information for ASOs, and to some extent for other oligonucleotides, aptamers remain a relatively unprecedented class from a toxicological point of view. When considering how to design an appropriate testing program and the class-related toxicological effects that should be considered, one needs to be fully aware of the available information for ASOs, but must not assume that what has been observed with ASOs as a class will also be true for aptamers, because aptamers differ from ASOs in length, structure, chemical composition, and PEG conjugation (usually).

The toxicological properties of ASOs have been extensively described, investigated, and published, even in complete reviews (32, 41, 42). This body of work is thorough and addresses program design considerations as well as specific findings in toxicology studies and how the chemical composition of an ASO can appreciably influence the toxicological properties of these molecules. Less information on other classes of oligonucleotides is available in the literature for immunostimulatory oligonucleotides, siRNAs, gene therapy, and other partially or completely nucleic acid–based molecules. From this literature, three major oligonucleotide class effects have been widely described. These include polyanion effects, stimulation of innate immunity, and tissue accumulation of oligonucleotide material. We briefly review these class effects below.

Polyanion effects result from nonspecific off-target protein interactions that may affect protein function and typically occur at high concentrations. The specific toxicological manifestations that have been associated with this category of effects include activation of the alternative pathway of complement (C′) resulting in pseudohypersensitivity responses and inhibition of the coagulation pathway resulting in prolongation of measured coagulation and clotting times. Complement activation has been ascribed to ASOs interacting with Factor H, a regulatory protein within the alternative pathway of C′ (43). When Factor H function is inhibited, the result is spontaneous activation of the alternative pathway. This can be measured by either C′ protein consumption (total hemolytic complement or CH50) or by measuring the split products from selected C′ pathway proteins that are formed upon activation (C3a and Bb) (42). Complement activation has been described predominantly in nonhuman primates and to a lesser extent in humans. Complement activation tends to occur acutely and is a threshold-dependent and concentration-related effect. Once triggered, C′ activation results predominantly in a cardiovascular syndrome characterized by hypotension and tachycardia, and when severe, can produce cardiovascular collapse and death in monkeys (44).

Anticoagulation has been described mainly in nonhuman primates and humans, and it has been attributed to low-affinity interactions and inhibition of the tenase complex of the coagulation cascade, resulting in a measurable prolongation of coagulation times, especially the activated partial thromboplastin time (41, 45). Whereas this phenomenon has been both widely described and mechanistically investigated, it is generally a modest effect and has, to our knowledge, never been associated with observed bleeding and may indeed be more of a laboratory observation than a true functionally relevant effect on hemostasis.

Another category of oligonucleotide class effects that has been extensively described is stimulation of the innate immune system. When originally described with first-generation ASOs, this effect was predominantly seen in rodents and was associated with the CpG motif within

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single-stranded DNA and a specific interaction with Toll-Like Receptor 9 (TLR 9). Research in this field subsequently exploded, and the use of designed oligonucleotides that were specifically optimized for this interaction resulted in a new class of intentionally immunostimulatory oligonucleotides (46, 47). In addition to the CpG–TLR 9 interaction, RNA-based molecules can stimulate other TLRs, specifically TLRs 3, 7, and 8, and elicit similar immunostimulatory effects (46). These interactions can lead to undesired immune stimulation that manifest as hyperplasia in lymphoid organs and mononuclear cell infiltrates in nonlymphoid organs (41). Cytokine release can be measured as a biomarker of these effects, and the specific pattern of cytokines can be influenced by the type of immunostimulatory molecule.

Lastly, the accumulation of oligonucleotide in certain tissues and cells has been repeatedly described. This phenomenon can be quantitatively measured (see above) and qualitatively visualized by immunohistochemistry, in situ hybridization, or standard hematoxylin- and eosin-stained tissue sections and histopathology (32, 48). The accumulated material has been morphologically described with the term basophilic granulation, and it is generally observed most prominently in tissue macrophages and renal proximal tubular epithelium. The presence of basophilic granules is generally not associated with adverse effects in the associated cells and tissues, but when it is present at very high concentrations, especially within the renal proximal tubular epithelium, degenerative change can occur along with measurable organ dysfunction (41).

These three categories of oligonucleotide class effects are not the only toxicities that have been described with oligonucleotides, but they are the dominant findings that should be carefully assessed when developing any oligonucleotide therapeutic. With this background information as well as the pharmacological and DMPK characteristics of aptamers described above, the following are some general concepts to consider in the safety assessment of aptamer therapeutics. First, although there are excellent descriptions of the toxicities observed with ASOs and related molecules, it is not appropriate to assume that aptamers possess the same properties. Aptamers differ appreciably in their length, structures, chemical compositions, and PEG conjugation, and these differences can dramatically change the properties relative to precedent oligonucleotides.

Second, pharmacological species restriction is commonly observed and is generally related to the degree of conservation of the target protein between species. It is common for an aptamer targeted to a human protein to have pharmacological cross-reactivity in nonhuman primates, but not in rodents or other preclinical species. This has implications in the selection of appropriate test species for toxicology testing, because it is strongly preferred to have at least one pharmacologically responsive test species in toxicology testing programs. It is our strategy to test aptamers in toxicology studies in two species, typically the cynomolgus monkey and the albino rat. In this paradigm, the nonhuman primate is sensitive to both target-related pharmacologically mediated effects and off-target mediated effects, whereas the rodent is typically susceptible to only nontarget-related effects.

Third, the site of action for aptamers is typically in the plasma or interstitial fluid compartments, and the pharmacodynamic effects of the aptamer can often be monitored with a blood-based measure of activity (depending on the target and target biology). It is therefore appropriate to measure plasma concentrations as the most relevant compartment to determine exposure and concentration-effect relationships, at least for pharmacologically related effects. This is in contrast to ASOs, siRNAs, and other oligonucleotides that act intracellularly, where the pharmacologically important concentrations are tissue and intracellular concentrations.

Fourth, the dose route and regimen of administration can vary dramatically between aptamers, depending on the aptamer design and intended use. Toxicology studies should reflect the intended human use. Lastly, dose selection criteria for toxicology studies should be performed as

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with any other modality, but it is common for the maximum achievable dose to be limited by maximum feasible dose considerations, especially with subcutaneous administration. As with other PEGylated molecules, the viscous nature of PEG at high concentrations tends to dominate the physical properties of dosing solutions and limit the maximum feasible concentration that can be injected. The maximum injectable concentration multiplied by the maximum allowable volume for injection often establishes the highest achievable dose in toxicology studies. It is important to note that by convention, we have expressed dose on the basis of oligonucleotide weight, exclusive of PEG. It is typical for the PEG component of a therapeutic aptamer to be approximately fourfold the mass of the oligonucleotide component. Therefore, for example, an aptamer administered at 10 mg/kg (oligonucleotide weight) has a total mass dose of ~50 mg/kg (total weight). This is important to note when one considers PEG-related effects and the PEG-equivalent doses.

ICH: International Conference on Harmonization

We have tested aptamers entered into development in standard International Conference on Harmonization (ICH)–recommended genotoxicity studies (typically the Ames bacterial mutagenicity assay, the human peripheral blood chromosomal aberrations assay, and the rat micronucleus assay). These studies have been uniformly negative for evidence of genotoxic effects. We have additionally tested aptamers in ICH-guided safety pharmacology studies that have typically included a hERG K-channel conductance study in vitro, a central nervous system pharmacological evaluation in the rat, and a cardiovascular and respiratory pharmacological evaluation in the telemetry-instrumented cynomolgus monkey. These studies have also been uniformly negative for meaningful safety pharmacology effects on these organ systems.

Our observations of general toxicology studies to date with five separate early development toxicology programs have demonstrated mostly stereotypical findings that have been common among the programs and considered to be class-based properties. The most consistent finding, observed in both rats and cynomolgus monkeys, in every program described to date is the accumulation of drug-related material in mononuclear phagocytes (macrophages and modified macrophages). This is a histological observation that is dose and time related. With PEGylated aptamers, the histological appearance is one of clear vacuoles in the cytoplasm of macrophages (**Figure 6**). The most commonly affected tissues are the liver, spleen, and lymph nodes, but almost any tissue with resident or infiltrating macrophages can be affected. In repeated-dose administration studies, this finding is typically first observed at daily doses between 1 and 10 mg/kg/day, and the magnitude (histological severity scores) and extent of distribution are dose related. Vacuolation of tissue macrophages has been described with other PEGylated products, and the vacuolar appearance of the cells is attributed to the presence of PEG in phagocytic vacuoles (34, 49, 50). We have confirmed that vacuoles contain drug-related material with immunohistochemical stains using

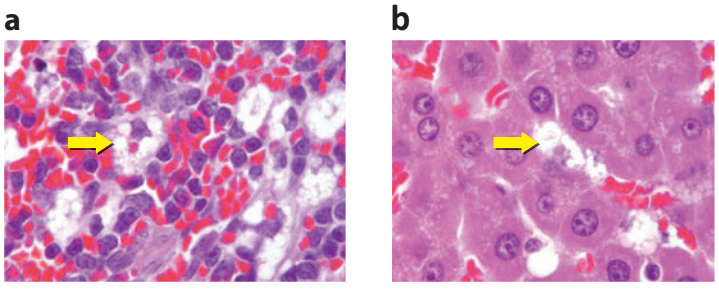

Figure 6

Photomicrographs of spleen (*a*) and liver (*b*) from a rat treated with a 40-kDa PEGylated aptamer for 2 weeks. Histological findings of vacuolization of tissue macrophages (at 400× magnification) owing to the uptake of PEGylated oligonucleotide are indicated by yellow arrows.

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anti-PEG antibody and with in situ hybridization using a complementary probe to the aptamer that has been administered (data not shown). In contrast, when un-PEGylated aptamers are administered at very high doses, drug-related material is observed in tissue macrophages and renal proximal tubular epithelium, but the histological appearance is of basophilic granules rather than vacuoles. This finding is analogous to the basophilic granules that have been extensively described with ASOs (41).

NoAEL: no-adverse-effect level or the highest dose tested that produces no adverse effects

Both vacuolation and basophilic granules are generally not associated with any other changes in the affected cells and tissues, so this finding is usually considered to represent the simple presence of drug-related material and is considered to be non-adverse. We have, however, on occasion observed at very high doses, and with marked accumulation of vacuoles in Kupffer cells of the liver, evidence of hyperplasia of Kupffer cells. When this type of reactive change is observed, we have considered this an adverse effect and use this type of finding as the basis for establishing a no-adverse-effect level (NoAEL). The presence of vacuoles or basophilic granules in tissue macrophages is slowly reversible upon cessation of dose administration. Partial resolution is generally observed following a 4-week dose-free recovery period, and substantial resolution is seen following a 13-week recovery period. The morphological evidence of slow resolution is interpreted as representing the process of degradation of aptamers within the phagocytic vacuoles of tissue macrophages followed by the clearance of material, as evidenced by the distribution and elimination of radiolabeled material in biodistribution and mass balance studies.

Other drug-related findings have been less consistently observed. Slight decreases in red blood cell mass accompanied by slight decreases in red cell reticulocytes have been observed at higher doses in repeated-dose administration studies in the rat. This finding has sometimes been associated with slight decreases in bone marrow cellularity and an accompanying finding of vacuolation of bone marrow macrophages. It is not clear if there is a relationship between these two findings.

We have on just one occasion in a single-dose IV infusion toxicology study in cynomolgus monkeys seen modest dose-related prolongation of the activated partial thromboplastin time, but this same effect was not repeated in a subsequent repeated-dose study at similar doses. Likewise, we have generally not seen C′ activation in cynomolgus monkey studies except for one instance with an all-DNA, un-PEGylated aptamer administered at extremely high doses ($\geq$600 mg/kg/day) and with plasma concentration in excess of 300 μg/mL, where modest increases in Bb and C3a were observed. Thus the polyanion effects that were prominently and consistently described with first-generation ASOs have been infrequent and negligible with the aptamers that have been evaluated in nonclinical studies to date. This difference may in part be explained by the lack of, or paucity of, phosphorothioate substitutions, a particular chemistry that is known to contribute to the polyanion properties of ASOs (40).

In one instance with a 40-kDa PEGylated aptamer administered by daily infusion at very high doses (>140 mg/kg/day oligonucleotide weight) and very high associated plasma concentrations ($\approx$100–200 μg/mL), we have observed a syndrome of plasma volume expansion and commensurate decreases in plasma components (such as serum proteins and blood cells). Such a change is not surprising with the large molecular mass and hydrophilicity of PEG that acts osmotically to draw water into the plasma compartment when plasma concentrations of PEG are very high. This effect has not been observed at dosages of 100 mg/kg/day or lower by any regimen of administration.

Overall, the general toxicology profile of therapeutic aptamers has been quite predictable and favorable through subchronic administration studies of up to 13 weeks in duration (longer-duration systemic administration toxicology studies have not yet been conducted). NoAELs are generally in the 25–100 mg/kg/day range, and have consistently demonstrated wide safety margins relative to the known or expected pharmacologically active doses in animal and humans.

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Table 3 Therapeutic Aptamers in Clinical Use or Clinical Development as of June 2009

Compound ID	Therapeutic Target	Disease Indication	Route/Method Administration	Clinical Phase	Selected References
Macugen™ (Pegaptanib Sodium)	VEGF	Macular degeneration	Intravitreal	Market	(51, 52)
ARC1779	von Willebrand factor	Thrombotic microangiopathy Adjunct to carotid endarterectomy	Intravenous infusion	Phase 2 Phase 2	(25, 40)
REG1	Factor IXa	Coronary artery bypass Percutaneous coronary intervention	Intravenous bolus	Phase 2 Phase 2	(53–55)
AS1411	Nucleolin	Acute myeologenous leukemia Renal cell carcinoma	Intravenous infusion	Phase 2 Phase 2	(56–58)
E10030	PDGF-b	Macular degeneration	Intravitreal	Phase 1	(59–60)
ARC1905	Complement factor 5	Macular degeneration	Intravitreal	Phase 1	(61–62)
NU172	Thrombin	Coronary artery bypass	Intravenous infusion	Phase 1	(63)

DEVELOPMENT STATUS, THERAPEUTIC POTENTIAL, AND FUTURE CHALLENGES AND OPPORTUNITIES FOR APTAMERS

As of June 2009, seven therapeutic aptamers were either approved for use as a marketed product (Macugen™) or in active clinical development (**Table 3**). All of the aptamers in clinical development have reported a favorable safety profile and varying levels of clinical activity. These examples in clinical development bode well for the continued success of therapeutic aptamers for acute or subacute intravenous use or local (especially intravitreal) administration applications. The next generations of therapeutic aptamers, yet to enter clinical development, are designed to have long plasma half-lives and the potential for intermittent SC delivery, and are intended for long-term therapy. The nonclinical pharmacology and PK profiles for molecules with these properties have been quite promising. Early subchronic toxicology data with repeated IV or SC dose administration is also promising, although more extensive testing is still needed to more fully define their chronic safety. What is still lacking is clinical experience with therapeutic aptamers intended for chronic intermittent administration. If competitive profiles in that setting can be demonstrated, then there will be a plethora of therapeutically relevant opportunities for this technology, much in the way that therapeutic antibodies have become a powerful therapeutic modality.

SUMMARY POINTS

1. Aptamers are structured oligonucleotides that bind to proteins with high affinity and specificity and thereby modulate protein function in much the same manner as an antibody.
2. Significant advances in selection pool design and postselection optimization of aptamers through chemical modification and PEG conjugation has significantly improved the therapeutic properties of aptamers.

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3. Advances in bioanalytical techniques have allowed for detailed study of the in vitro and in vivo DMPK properties of therapeutic aptamers and has informed the further optimization of DMPK properties.
4. Aptamers have been largely devoid of the toxicology class effects previously reported with early antisense oligonucleotides.
5. The principal finding in toxicology studies of therapeutic aptamers has been the histologically visible accumulation of oligonucleotide in mononuclear phagocytes throughout the body. This finding has generally not been considered adverse, and the therapeutic aptamers tested to date have shown good safety margins between the pharmacologically effective dose and the toxicologically established no-adverse-effect levels.
6. Several therapeutic aptamers have progressed into mid- or late-stage clinical trials, and this new class of drugs has shown excellent characteristics for local and acute systemic administration applications. Safety and efficacy under chronic systemic administration conditions has yet to be demonstrated, but strong preclinical profiles support the advancement of therapeutic aptamers into those applications.

DISCLOSURE STATEMENT

The authors have all been employees of or consultants to Archemix Corp., a biotechnology company dedicated to discovery and development of aptamer therapeutics, and as such have an interest in the successful development of this class of drugs. This interest has the potential to affect their objectivity in this review.

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LITERATURE CITED

1. Brody EN, Gold L. 2000. Aptamers as therapeutic and diagnostic agents. *Rev. Mol. Biotechnol.* 74:2–13
2. Proske D, Blank M, Buhmann R, Resch A. 2005. Aptamers: basic research, drug development, and clinical applications. *Appl. Microbiol. Biotechnol.* 69:367–74
3. Pestourie C, Tavitian B, Duconge F. 2005. Aptamers against extracellular targets for in vivo applications. *Biochemie* 87:921–30
4. Nimjee SM, Rusconi CP, Harrington RA, Sullenger BA. 2005. The potential of aptamers as anticoagulants. *Trends Cardiovasc. Med.* 15:41–45
5. Lee JF, Stovall GM, Ellington AD. 2006. Aptamer therapeutics advance. *Curr. Opin. Chem. Biol.* 10:282–89
6. Osborne SE, Matsumura I, Ellington AD. 1997. Aptamers as therapeutic and diagnostic reagents: problems and prospects. *Curr. Opin. Chem. Biol.* 1:5–9
7. Keefe AD, Cload ST. 2008. SELEX with modified nucleotides. *Curr. Opin. Chem. Biol.* 12:448–56
8. Marshall KA, Ellington AD. 2000. In vitro selection of RNA aptamers. *Methods Enzymol.* 318:193–214
9. Kaur G, Roy I. 2008. Therapeutic applications of aptamers. *Expert Opin. Investig. Drugs* 17:43–60
10. Wilson C. 2008. Aptamer opportunities and challenges. In *Antisense Drug Technology, Principles, Strategies and Applications*, ed. S Crooke, pp. 773–99. Boca Raton, FL: Taylor & Francis
11. Tuerk C, Gold L. 1990. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* 249:505–10

Annu. Rev. Pharmacol. Toxicol. 2010.50:237-257.

12. Ellington AD, Szostak JW. 1990. In vitro selection of RNA molecules that bind specific ligands. *Nature* 346:818–22
13. Sousa R, Padilla R. 1995. A mutant T7 RNA polymerase as a DNA polymerase. *EMBO J.* 14:4609–21
14. Vater A, Klussmann S. 2003. Toward third-generation aptamers: Spiegelmers and their therapeutic prospects. *Curr. Opin. Drug Discov. Dev.* 6:253–61
15. Clauss S, Gross O, Kulkarni O, Avila-Ferrufino A, Radomska E, et al. 2009. Ccl2/Mcp-1 blockade reduces glomerular and interstitial macrophages but does not ameliorate renal pathology in collagen4A3-deficient mice with autosomal recessive Alport nephropathy. *J. Pathol.* 218:40–47
16. Edvinsson L, Nilsson E, Jansen-Olesen I. 2007. Inhibitory effect of BIBN4096BS, CGRP(8–37), a CGRP antibody and an RNA-Spiegelmer on CGRP induced vasodilatation in the perfused and non-perfused rat middle cerebral artery. *Br. J. Pharmacol.* 150:633–40
17. Shearman LP, Wang SP, Helmling S, Stribling DS, Mazur P, et al. 2006. Ghrelin neutralization by a ribonucleic acid-SPM ameliorates obesity in diet-induced obese mice. *Endocrinology* 147:1517–26
18. Helmling S, Maasch C, Eulberg D, Buchner K, Schröder W, et al. 2004. Inhibition of ghrelin action in vitro and in vivo by an RNA-Spiegelmer. *Proc. Natl. Acad. Sci. USA* 101:13174–79
19. Leva S, Lichte A, Burmeister J, Muhn P, Jahnke B, et al. 2002. GnRH binding RNA and DNA Spiegelmers: a novel approach toward GnRH antagonism. *Chem. Biol.* 9:351–59
20. Bailon P, Won CY. 2009. PEG-modified biopharmaceuticals. *Expert Opin. Drug Deliv.* 6:1–16
21. Jain A, Jain SK. 2008. PEGylation: an approach for drug delivery. A review. *Crit. Rev. Ther. Drug Carrier Syst.* 25:403–47
22. Veronese FM, Mero A. 2008. The impact of PEGylation on biological therapies. *BioDrugs* 22:315–29
23. Veronese FM, Pasut G. 2005. PEGylation, successful approach to drug delivery. *Drug Discov. Today* 10:1451–58
24. Hamidi M, Azadi A, Rafiei P. 2006. Pharmacokinetic consequences of pegylation. *Drug Deliv.* 13:399–409
25. Diener J, Lagassé D, Duerschmied D, Merhi Y, Tanguay JF, et al. 2009. Inhibition of von Willebrand Factor-mediated platelet activation and thrombosis by anti-von Willebrand Factor A1-domain aptamer ARC1779. *J. Thromb. Haemost.* 7:1155–62
26. Levin AA, Yu RZ, Geary RS. 2008. Basic principles of the pharmacokinetics of antisense oligonucleotide drugs. See Ref. 10, pp. 183–215
27. Wu H, MacLeod AR, Lima WF, Crooke ST. 1998. Identification and partial purification of human double strand RNase activity. A novel terminating mechanism for oligoribonucleotide antisense drugs. *J. Biol. Chem.* 273(5):2532–42
28. Hutabarat RM, Chung J, Lewis SL, Cai L, McCauley TG, Bouchard P. 2008. Metabolic stability, metabolite identification and nuclease cleavages identification by LC/MS/MS during aptamer lead series optimization. Presented at National Biotechnology Conference
29. Drolet DW, Nelson J, Tucker CE, Zack PM, Nixon K, et al. 2000. Pharmacokinetics and safety of an anti-vascular endothelial growth factor aptamer (NX1838) following injection into the vitreous humor of rhesus monkeys. *Pharm. Res.* 17:1503–10
30. McCauley TG, Kurz JC, Merlino PG, Lewis SD, Gilbert M, et al. 2006. Pharmacologic and pharmacokinetic assessment of anti-TGFbeta2 aptamers in rabbit plasma and aqueous humor. *Pharm. Res.* 23:303–11
31. Leeds JM, Henry SP, Geary R, Burckin T, Levin AA. 2000. Comparison of the pharmacokinetics of subcutaneous and intravenous administration of a phosphorothioate oligodeoxynucleotide in cynomolgus monkeys. *Antisense Nucleic Acid Drug Dev.* 10:435–41
32. Farman CA, Kornbrust DJ. 2003. Oligodeoxynucleotide studies in primates: antisense and immune stimulatory indications. *Toxicol. Pathol.* 31(Suppl):119–22
33. Geary RS, Yu RZ, Watanabe T, Henry SP, Hardee GE, et al. 2003. Pharmacokinetics of a tumor necrosis factor-alpha phosphorothioate 2′-O-(2-methoxyethyl) modified antisense oligonucleotide: comparison across species. *Drug Metab. Dispos.* 31(11):1419–28
34. U.S. Food and Drug Administration. 2004. Macugen, Pegaptanib Sodium. FDA Pharmacology Review. **http://www.accessdata.fda.gov/scripts/cder/drugsatfda/**
35. Boomer RM, Lewis SD, Healy JM, Kurz M, Wilson C, McCauley TG. 2005. Conjugation to polyethylene glycol polymer promotes aptamer distribution to healthy and inflamed tissues. *Oligonucleotides* 15:183–95

Annu. Rev. Pharmacol. Toxicol. 2010.50:237-257.

36. Phillips JA, Craig SJ, Bayley D, Christian RA, Geary R, Nicklin PL. 1997. Pharmacokinetics, metabolism, and elimination of a 20-mer phosphorothioate oligodeoxynucleotide (CGP 69846A) after intravenous and subcutaneous administration. *Biochem. Pharmacol.* 54(6):657–68
37. Graham MJ, Crooke ST, Monteith DK, Cooper SR, Lemonidis KM, et al. 1998. In vivo distribution and metabolism of a phosphorothioate oligonucleotide within rat liver after intravenous administration. *J. Pharmacol. Exp. Ther.* 286(1):447–58
38. Geary RS, Khatsenko O, Bunker K, Crooke R, Moore M, et al. 2001. Absolute bioavailability of 2′-O-(2-methoxyethyl)-modified antisense oligonucleotides following intraduodenal instillation in rats. *J. Pharmacol. Exp. Ther.* 296(3):898–904
39. DeAnda A Jr, Coutre SE, Moon MR, Vial CM, Griffin LC, et al. 1994. Pilot study of the efficacy of a thrombin inhibitor for use during cardiopulmonary bypass. *Ann. Thorac. Surg.* 58:344–50
40. Gilbert JC, DeFeo-Fraulini T, Hutabarat RM, Horvath CJ, Merlino PG, et al. 2007. First-in-human evaluation of anti von Willebrand factor therapeutic aptamer ARC1779 in healthy volunteers. *Circulation* 116:2678–86
41. Henry SP, Kim TW, Kramer-Strickland K, Zanardi TA, Fey RA, Levin AA. 2008. Toxicological properties of 2′-O-methoxyethyl chimeric antisense inhibitors in animals and man. See Ref. 10, pp. 327–63
42. Marquis JK, Grindel JM. 2000. Toxicological evaluation of oligonucleotide therapeutics. *Curr. Opin. Mol. Ther.* 2:258–63
43. Henry SP, Giclas PC, Leeds J, Pangburn M, Auletta C, et al. 1997. Activation of the alternative pathway of complement by a phosphorothioate oligonucleotide: potential mechanism of action. *J. Pharmacol. Exp. Ther.* 281:810–16
44. Henry SP, Beattie G, Yeh G, Chappel A, Giclas P, et al. 2002. Complement activation is responsible for acute toxicities in rhesus monkeys treated with a phosphorothioate oligodeoxynucleotide. *Int. Immunopharmacol.* 2:1657–66
45. Sheehan JP, Lan HC. 1998. Phosphorothioate oligonucleotides inhibit the intrinsic tenase complex. *Blood* 92:1617–25
46. Vollmer J, Krieg AM. 2008. Mechanisms and therapeutic applications of immune modulatory oligodeoxynucleotide and oligoribonucleotide ligands for toll-like receptors. See Ref. 10, pp. 747–72
47. Barchet W, Wimmenauer V, Schlee M, Hartmann G. 2008. Accessing the therapeutic potential of immunostimulatory nucleic acids. *Curr. Opin. Immunol.* 20:389–95
48. Goebl N, Berridge B, Wroblewski VJ, Brown-Augsburger PL. 2007. Development of a sensitive and specific in situ hybridization technique for the cellular localization of antisense oligodeoxynucleotide drugs in tissue sections. *Toxicol. Pathol.* 35:541–48
49. Bendele A, Seely J, Richey C, Sennello G, Shopp G. 1998. Short communication: renal tubular vacuolation in animals treated with polyethylene-glycol-conjugated proteins. *Toxicol. Sci.* 42:152–57
50. U.S. Food and Drug Administration. 2008. Cimzia, Certolizumab pegol. FDA Pharmacology Review. **http://www.accessdata.fda.gov/scripts/cder/drugsatfda/**
51. Ng EW, Shima DT, Calias P, Cunningham ET Jr, Guyer DR, Adamis AP. 2006. Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease. *Nat. Rev. Drug Discov.* 5:123–32
52. Ciulla TA, Rosenfeld PJ. 2009. Antivascular endothelial growth factor therapy for neovascular age-related macular degeneration. *Curr. Opin. Ophthalmol.* 20:158–65
53. Dyke CK, Steinhubl SR, Kleiman NS, Cannon RO, Aberle LG, et al. 2006. First-in-human experience of an antidote-controlled anticoagulant using RNA aptamer technology: a phase 1a pharmacodynamic evaluation of a drug-antidote pair for the controlled regulation of factor IXa activity. *Circulation* 114:2490–97
54. Chan MY, Rusconi CP, Alexander JH, Tonkens RM, Harrington RA, Becker RC. 2008. A randomized, repeat-dose, pharmacodynamic and safety study of an antidote-controlled factor IXa inhibitor. *J. Thromb. Haemost.* 6:789–96
55. Chan MY, Cohen MG, Dyke CK, Myles SK, Aberle LG, et al. 2008. Phase 1b randomized study of antidote-controlled modulation of factor IXa activity in patients with stable coronary artery disease. *Circulation* 117:2865–74
56. Bates PJ, Laber DA, Miller DM, Thomas SD, Trent JO. 2009. Discovery and development of the G-rich oligonucleotide AS1411 as a novel treatment for cancer. *Exp. Mol. Pathol.* 86:151–64

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57. Soundararajan S, Chen W, Spicer EK, Courtenay-Luck N, Fernandes DJ. 2008. The nucleolin targeting aptamer AS1411 destabilizes Bcl-2 messenger RNA in human breast cancer cells. *Cancer Res.* 68:2358–65
58. Ireson CR, Kelland LR. 2006. Discovery and development of anticancer aptamers. *Mol. Cancer Ther.* 5:2957–62
59. Jo N, Mailhos C, Ju M, Cheung E, Bradley J, et al. 2006. Inhibition of platelet-derived growth factor B signaling enhances the efficacy of anti-vascular endothelial growth factor therapy in multiple models of ocular neovascularization. *Am. J. Pathol.* 168:2036–53
60. Sennino B, Falcón BL, McCauley D, Le T, McCauley T, et al. 2007. Sequential loss of tumor vessel pericytes and endothelial cells after inhibition of platelet-derived growth factor B by selective aptamer AX102. *Cancer Res.* 67:7358–67
61. Biesecker G, Dihel L, Enney K, Bendele RA. 1999. Derivation of RNA aptamer inhibitors of human complement C5. *Immunopharmacology* 42:219–30
62. Kurz JC, Rottman JB, McCauley TM, Benedict C, Epstein D. 2005. In vitro and in vivo studies of a synthetic anti-C5 aptamer. Presented at 3rd Workshop on Complement Associated Diseases, Animal Models, and Therapeutics
63. Wagner-Whyte J, Khuri S, Olsen K, Hatala P, Boomer RM, et al. 2007. Discovery of an extremely potent aptamer direct thrombin inhibitor. ISTH Conference

Annu. Rev. Pharmacol. Toxicol. 2010.50:237-257.

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