Nucleic acid selection as a tool for drug discovery

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Nucleic acid selection has proven to be an extremely versatile technique for the isolation of aptamers that can bind tightly and specifically to target molecules. Aptamers can modulate cellular physiology and have begun to be developed for therapeutic applications. Aptamers have also begun to be used as research reagents for the detection and localization of proteins. Moreover, because nucleic acid selection can quickly yield compounds that have unique properties and that can be applied in unique roles, this technique should generally be used as a tool to augment drug discovery efforts.

In recent years several techniques have been devised for the synthesis and functional screening of large numbers of highly varied organic molecules. Libraries of peptides, antibodies or partially randomized proteins can be displayed on the surfaces of bacteriophage or cells, and variants with desirable binding or catalytic abilities can be iteratively selected and amplified[1,2]. Similarly, functional nucleic acids can be selected from random-sequence nucleic acid pools, a process variously known as SELEX (systematic evolution of ligands by exponential enrichment)[3], directed molecular evolution[4] or *in vitro* selection[5]. Finally, the large-scale parallel synthesis and evaluation of organic compounds, either singly or in groups, has become a popular alternative to more traditional medicinal chemistry programs[6].

The development of techniques for combinatorial biochemistry and chemistry has been driven in part by the vast investment – both in terms of time and money (currently estimated at ten years and >$475 million) – required to bring fewer than one lead compound in ten thousand to market. In particular, combinatorial methods can augment or complement drug discovery efforts both by increasing the diversity of available lead compounds and by honing the affinities, specificities and pharmacokinetics of lead compounds during the initial phases of discovery. However, the establishment of any new program in combinatorial biochemistry or chemistry is technically demanding, and it is often unclear when and where these different methods should be utilized and how they should be best integrated with other facets of the drug discovery process. Without undue prejudice, the authors' expertise can serve as a guide as to how one technique, nucleic acid selection, can be used to augment drug discovery efforts. Drawing on the recent literature, several topics will be covered: the mechanics of selection experiments; the efficacies of selected nucleic acid-binding species (also known as aptamers); the potential for therapeutic applications of aptamers; the use of aptamers for target validation and identification; and the use of aptamers as research reagents.

Nucleic acid selection

Several key steps are common to all selection methods for nucleic acid-binding species: first, generation of sequence diversity; second, selection of functional shapes; and third, amplification[4] (Figure 1). Initially, a large pool of sequence diversity is generated by chemical synthesis and then amplified or transformed by enzymatic manipulations such as PCR or *in vitro* transcription. Although nucleic acids are traditionally thought of as messenger 'tapes', each different nucleic acid sequence will fold into a distinct three-dimensional shape[7]. Frequently, some of the multitude of nucleic acid shapes will be chemically complementary to the surface of a target molecule. A few examples of the diverse

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secondary structures that nucleic acids are known to assume are illustrated in Figure 2. Functional nucleic acids that contain stem-loops, stem-bulges, multi-arm junctions, pseudoknots or even quadruplexes have all been isolated by *in vitro* selection.

In the actual selection step, the population of nucleic acid sequences and shapes is mixed with a target and then nucleic acid–target complexes are sieved from the population by any one of a variety of methods, such as filtration or affinity chromatography. The binding species are then eluted and the selected population is amplified by procedures such as PCR or isothermal amplification[8]. This selection and reamplification of a population of molecules is often referred to as a round, cycle or generation. Multiple generations are typically required to isolate the best (highest affinity, greatest specificity) binding species. As many amplification procedures are inherently mutagenic, sequences that may not have been present in the original population can also be selected. Although the basic steps of nucleic acid selection are relatively simple, it will become apparent that the overall process is extremely robust and can be successfully applied to numerous and varied target molecules.

Generating the sequence pool

One of the reasons nucleic acid-selection experiments have proven versatile, and one that is a unique advantage over other combinatorial techniques, is that the chemistry and size of the random-sequence population can be easily manipulated. The initial random-sequence pool is typically generated by chemical DNA synthesis and contains a core of randomized nucleotides that are flanked by constant regions, which are required for enzymatic amplification. The constant regions may include promoter sequences, necessary for *in vitro* transcription of RNA[2,4], and specific restriction sites that aid cloning of selected species[9]. Thus, the original single-stranded DNA pool can be readily converted into either a double-stranded DNA pool or a single-stranded RNA pool. Unnatural nucleotides can also be included in the DNA or RNA polymerization reactions in place of natural nucleotides to generate nucleic acid pools with substantially different chemistries[10–13]. Similarly, the termini of random sequence pools can be conjugated to compounds that will augment their binding or catalytic capabilities[14].

The random regions in the nucleic acid sequence can either be a partially mutagenized version of a known sequence or can be fully randomized. Partially randomized pools are obtained by 'doping' a constant sequence mixture with non-wild-type residues; for example, a guanosine that is normally present in a sequence might be synthesized as a mixture of 70% guanosine, 10% adenosine, 10% thymidine and 10% cytidine. Doped sequence pools can be used to define rapidly either the nucleic acid secondary structure[15] or the nucleic acid–protein interactions[16]. Since a doped sequence pool often spans all possible single- through hextuple-substitutions of a given wild-type sequence[17,18], selection experiments can, in essence, simultaneously probe a huge array of mutations for function. Doped sequence pools can also be used to identify nucleic acids that have binding properties similar to those of a wild-type sequence[19,20]. Such 'unnatural' binding sites can then be used to probe nucleic acid function. For example, aptamers that bind to the elongation factor SelB have been isolated from a doped sequence pool: SelB is responsible for the specific incorporation of selenocysteine across from opal stop codons in enzymes such as formate dehydrogenase[21,22], but its precise mechanism of action is unclear. The 'unnatural' SelB-binding sequences were incorporated into the formate dehydrogenase gene and the resultant constructs were assayed for their ability to incorporate selenocysteine. These experiments confirmed that the sequence and structure of an RNA stem-loop that formed adjacent to the opal stop codon were essential for efficient and selective amino acid insertion.

Completely random pools, by contrast, can be used for *de novo* isolation of functional molecules with novel properties. A fully random sequence pool typically contains from 30 to 200 randomized residues and spans anywhere from 10^{13} to 10^{17} different nucleic acid species. To fully appreciate these figures, it should be recognized that the diversity of a nucleic acid population is 10^{1}–10^{5}-times greater than the diversity of some of the largest phage display populations that have been generated, and 10^{4}–10^{8}-times greater than the estimated number of antibodies that comprise the human immune system. While such comparisons are impressive, they are not necessarily informative: for example, it is difficult to know a priori whether a nucleic acid library that spans 100 residues contains more or less functional species than a dodecamer peptide library or a benzodiazepine library, or whether the functional species are better or worse. Nonetheless, as nucleic acid libraries contain more conformational shapes than other libraries, they are also likely to contain a better representation of the structural gradations between shapes. Given that interactions with target molecules are frequently controlled by bonding and steric

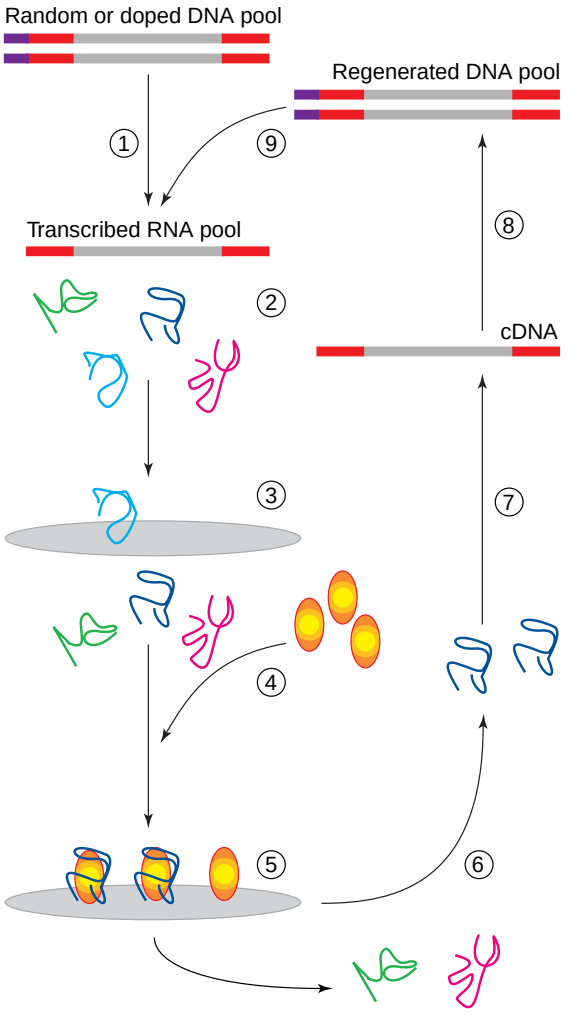

Figure 1. *Aptamer selection scheme showing one round of an* in vitro *selection against a protein target using co-retention onto a filter. The chemically synthesized DNA pool (1) includes promoter sequences (purple) that are not present in the RNA pool and known primer sequences (red). The transcribed RNA molecules (2) fold into unique shapes (shown by various colored strands) and in a negative selection step (3), RNA species that bind nonspecifically to the matrix can be removed from the population. (4) RNA species that flow through the filter are mixed with a protein target (yellow beads) and on a second pass through a filter (5), proteins are uniformly bound along with protein-bound RNA species. (6) The bound RNA species are eluted and following the regeneration of the RNA pool via reverse transcription (7), PCR (8) and* in vitro *transcription (9), the round is repeated. Multiple rounds of selection and amplification are carried out until a desired end point, usually a low* K_d*, is achieved.*

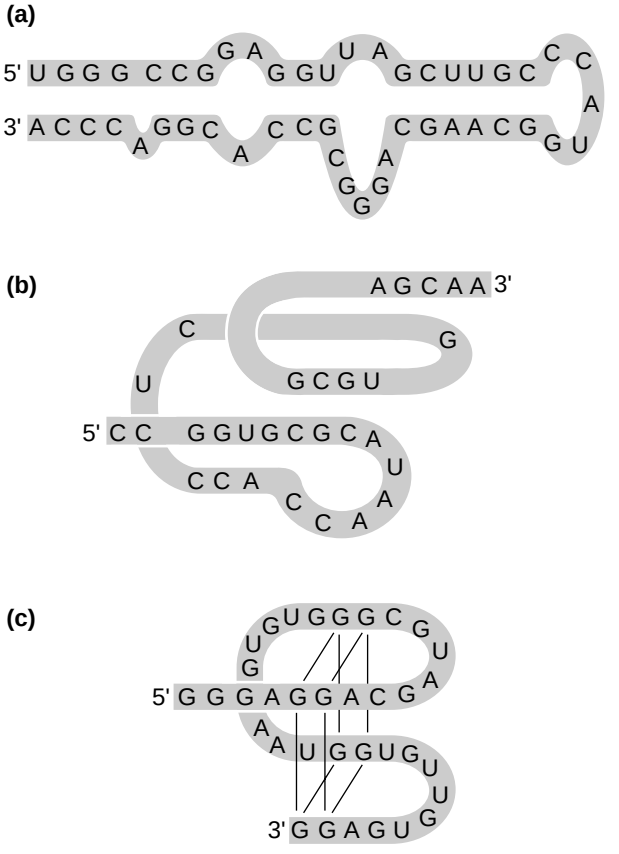

Figure 2. *Secondary structures of aptamers. (a) An aptamer selected to bind autoimmune antibodies raised against the acetylcholine receptor[55]. Internal loops and side bulges are embedded within a long stem loop. (b) An anti-cyanocobalamin aptamer that forms a pseudoknot[36]. (c) An anti-IgE aptamer that contains a central G-quartet stack[54].*

effects in the order of tenths of Angstroms, a more complete representation of minute structural gradations may be essential for the identification of truly high affinity species.

Partitioning of the pool

Because of the simplicity of the selection procedure, huge nucleic acid populations with diverse chemistries can be quickly matured into specific compounds with tailored affinities and specificities. First, the stringency of nucleic acid-selection experiments can be controlled by varying the stringency of individual rounds of selection or by varying the number of rounds of selection. For example, continued rounds of selection have been shown, by comparative methods, to be effective in increasing binding affinity of aptamers for their targets – as has been long predicted by

theoretical techniques[23]. Second, as mutation occurs or can be induced during amplification, aptamers that may initially have only modest binding affinities can continue to mutate and 'hill climb' to functional optima. The progressive maturation of aptamer affinity by reselection contrasts with the laborious optimization of pharmacophores by iterative synthesis and assay for activity. Third, the specificity profile of an aptamer can be modulated by the inclusion of 'negative selection' steps. For example, Jenison *et al.* isolated aptamers that could specifically bind to the drug theophylline[24]. During their selection process, the researchers removed nucleic acid species that bound caffeine (which differs from theophylline by only a single methyl group) before selection of species that bind theophylline. As a result, the anti-theophylline aptamers discriminated against caffeine by almost 10,000-fold, a binding specificity that was better than that achieved by comparable monoclonal antibodies.

Advantages of aptamers

In addition to the advantages inherent in the selection procedure, it has become apparent that the products of the procedure, the aptamers themselves, have several characteristics that are highly desirable for drug discovery: aptamers can acquire a wide range of targets, they can bind to their targets with high affinities and they can discriminate between closely related, noncognate targets.

Target range

A wide variety of molecules have so far been targeted by *in vitro* selection experiments and have yielded specific nucleic acid-binding sequences. Perhaps the smallest target yet attempted has been a chelated zinc molecule. The anti-zinc chelate aptamers contained a structured binding pocket that was duly protected by zinc from chemical modification[25,26]. Many small organic molecules, such as dyes or cofactors with molecular weights from 100 to 1,000, have also proven to be excellent targets for selection[5,27]. In general, targets that contain positive charges or heterocycles seem to yield tighter molecular complexes than targets that lack these features. For example, a selection procedure that targeted the amino acid arginine returned aptamers that formed complexes with dissociation constants in the nanomolar range[28], while a selection that targeted the amino acid valine returned aptamers that formed complexes with dissociation constants in the millimolar range[29]. Peptides such as substance P (Ref. 30) or the arginine-rich motif of the HIV-1 Rev protein[31] have been used to elicit aptamers and, again, the presence of positive charges on the peptides appears to have enhanced the interactions with the polyanionic aptamers.

Literally hundreds of proteins have been targeted by selection and have yielded anti-protein aptamers[32]. These proteins include nucleic acid-binding proteins, such as T4 DNA polymerase and HIV-1 Rev, but also non-nucleic-acid-binding proteins, such as cytokines, immunoglobulins and kinases. In addition, supramolecular structures can be the targets of selection; for example, aptamers that can bind to the coat of the Rous Sarcoma Virus have been isolated[33]. Most recently, aptamers have been selected that can specifically bind to wounded tissue. Overall, aptamers can not only recognize the same range of target molecules as antibodies but they can also be selected against targets that might be difficult to employ as haptens, such as toxins[34] or prions[35].

Target binding

Aptamers frequently bind their targets with very high affinities, rivaling the affinities of monoclonal antibodies[5]. Aptamers selected to bind small organic molecules form complexes that have dissociation constants in the micromolar to submicromolar range. For example, an aptamer selected to bind vitamin B_{12} formed a complex with a dissociation constant of 320 nM (Ref. 36). Aptamers selected to bind proteins form complexes that frequently have dissociation constants in the nanomolar range[32]. In fact, anti-vascular endothelial growth factor (VEGF) aptamers have been found to form complexes with VEGF that have dissociation constants as low as 0.2 nM (Ref. 37) and modified anti-VEGF aptamers have been generated that bind with an order of magnitude better[36]. A review by Gold *et al.*[32] revealed that fewer than 15 of 50 selection experiments that targeted proteins, produced aptamers that complexed with their cognate ligands with dissociation constants >100 nM.

Target distinction

Aptamers frequently bind their targets with exquisite specificity. Just as the anti-theophylline aptamers could discriminate between targets that differed by only a single methyl group, anti-ATP aptamers have been shown to discriminate against dATP, which differs by just a single hydroxyl group. The structure of the anti-ATP aptamer has been solved: the structural basis for the discrimination between a 2′-hydroxyl and a 2′-hydrogen is the formation of hydrogen bonds to the 2′-hydroxyl by up to three juxtaposed aptamer bases. Similarly, Conrad *et al.*[38] selected anti-protein kinase C

(PKC) aptamers that could distinguish between PKC isozymes with 96% sequence homology. Even more surprisingly, aptamers selected to bind to wild-type thrombin do not interact with a variant of thrombin differing by just one amino acid[39], while a slightly different aptamer was selected to recognize both thrombin and its variant[40].

Therapeutic applications of aptamers

It has been suggested that aptamers have promise as potential nucleic acid pharmaceuticals[7]. However, the cost of their synthesis, their bioavailability and pharmacokinetics, and the means by which they will be delivered all remain daunting obstacles to immediate application. These obstacles are being steadily overcome by companies dedicated to the development of aptamers for the marketplace, such as NeXstar. Nonetheless, for most companies aptamers remain a curiosity. This situation will probably change in the near future because, for some targets, the unique properties and propensities of aptamers may well prove to be the best possible lead compounds. The therapeutic potential of aptamers can be gauged by examining their ability to inhibit biomedically relevant target molecules, their ability to modulate physiological function and their ability to be engineered for stability and other properties.

Target inhibition

Surprisingly, many of the aptamers selected to bind protein targets also inhibit the activities of those targets. For example, RNA and DNA aptamers have been selected that can bind to the reverse transcriptase (RT) of HIV-1 (Refs 41,42). The RNA aptamers folded into a pseudoknot structure and formed complexes with RT that have a K_d = 5 nM (Ref. 41). Similarly, the single-stranded DNA aptamers could form complexes with a K_d = 1 nM and could inhibit the enzyme's DNA-polymerase activity with a K_i = 0.3 nM (Ref. 42). An aptamer raised against avian myeloblastosis virus (AMV)-RT was isolated from an RNA pool that had an IC_{50} = 9 nM for polymerase activity.

Finally, an aptamer against Moloney murine leukemia virus (MMLV)-RT inhibited this enzyme's RNase H activity at a concentration of 25 nM. Each of these aptamers was specific for its cognate target: the anti-HIV-1 RT aptamers did not bind to AMV RT or MMLV RT; the anti-AMV-RT aptamer bound the MMLV RT 80-fold less effectively than AMV; and the anti-MMLV-RT aptamer formed a complex with AMV RT that had a K_d in the micromolar range. Non-nucleic-acid-binding proteins can also be effectively inhibited by aptamers. The human T-cell leukemia virus (HTLV) transcriptional activator Tax interacts with cellular transcription factors. Anti-Tax aptamers can specifically disrupt these protein–protein interactions[43]. Many additional examples with greater physiological relevance will be cited below.

Similar to antibodies, aptamers are selected solely on the basis of their ability to form a complex with a target molecule – without regard for which surface, cleft or epitope on the target molecule that will be bound. Thus, it is surprising that aptamers selected for their protein-binding abilities also frequently inhibit protein activities. To employ a different jargon, while only some antibodies that bind a target appear to be neutralizing antibodies, most aptamers that bind a target are neutralizing aptamers. One rationale for this observation is that antibodies have preformed binding cusps, but aptamers must evolve both the interactions with a target and a structure to support those interactions. Thus, antibodies have enough nascent structure to bind exposed epitopes, while the most prevalent and most effective aptamers will fold into compact structures that fit into the crevices or clefts on the surface of proteins. Moreover, because such crevices or clefts are frequently active or regulatory sites, anti-protein aptamers will frequently inhibit protein function.

An interesting consequence of the fact that aptamers fit into protein pockets is the observation that aptamers selected to bind to antibodies that are directed against the insulin receptor, mimicked an epitope on the insulin receptor[44]. In other words, the antibody had a pocket that fits around a surface feature on the receptor and, in order to fit into this pocket, the aptamers recreated some of the chemical and structural details of the original surface feature. These results also have implications for the development of novel, nucleic acid-based vaccines.

Improving aptamer affinity

Although the homing abilities of aptamers are impressive, modifications of selection techniques have been developed to increase the probability that selected species will tightly bind to and strongly inhibit their targets. To increase the affinity of aptamers for their targets, a crosslinking reagent can be incorporated into the pool. For example, Jensen *et al.*[13] selected aptamers that could form covalent bonds to HIV-1 Rev from a random sequence pool in which the photoreactive nucleotide 5′-iodouridine was incorporated in place of uridine. The modified RNA pool was mixed with Rev and the reaction mixture was irradiated with UV light. Immobilized aptamers were then isolated by electrophoresis

on a denaturing polyacrylamide gel. The Rev protein was digested away from the isolated nucleoprotein complex with proteinase K and the remaining nucleic acid was amplified and reselected. The resultant anti-Rev aptamers could specifically photocrosslink to Rev. In addition, some of the selected species unexpectedly appeared to bind extremely tightly to the Rev protein even in the absence of light, perhaps via an amino acid-catalyzed Michael addition. To direct the binding of an aptamer to the active site of a protein, a known ligand or inhibitor of a protein can be appended to an oligonucleotide pool prior to or following selection. For example, valyl phosphonate (ValP) is a mechanism-based inhibitor of human neutrophil elastase (hNE) that forms a covalent adduct upon reaction. Valyl phosphonate was conjugated to a DNA oligonucleotide that was in turn designed to make a hydrogen bond to a constant sequence region of an RNA pool[45]. Tightly binding aptamers were again selected and the inhibition constant of the aptamer conjugates for elastase cleavage activity on a peptide substrate were found to be in the order of 10 nM, while ValP alone inhibited cleavage 100-fold less effectively. A similar selection was also carried out in which the ValP–oligonucleotide conjugate was splinted to a DNA pool (i.e. bound via base-pair interactions between the oligonucleotide portion of the conjugate and one primer region of the DNA pool)[46].

Finally, an unmodified DNA aptamer selected to bind hNE was conjugated following selection to a competitive tetrapeptide inhibitor (*N*-methoxysuccinyl-Ala-Ala-Pro-Val) via a flexible linker. The binding and inhibitory abilities of both the aptamer and the peptide were improved by the union, because the covalent linker enforced cooperative interactions between what would otherwise have been discrete binding species and sites. The K_i of the peptide–aptamer conjugate was 28 nM, while the K_i of the aptamer alone was four orders of magnitude lower[14]. Taken together, these examples serve to demonstrate how the selection process itself can be used to engineer the affinities, specificities and inhibitory abilities of aptamers. By contrast, it would be difficult to derive peptides from phage display selections, or antibodies from an immunization procedure, that included either an unnatural amino acid or a conjugated inhibitor. The three different strategies that have so far been used to improve or direct binding are summarized in Figure 3.

Aptamer efficacy

Aptamers not only inhibit the activities of proteins but they can also modulate cellular or organismal physiology. Although we have not considered the problems associated with the delivery of aptamers in detail, the following examples are roughly grouped according to whether the aptamers target intracellular proteins (and thus might be ensconced within liposomes or expressed from an integrated retroviral vector) or target extracellular proteins (and thus might be orally administered or injected into the bloodstream).

Aptamers that target intracellular proteins have been shown to affect both normal metabolism and disease states. For example, RNA aptamers selected to bind E2F, a transcription factor essential for cell cycle progression, could induce growth arrest when injected into human diploid fibroblast cells[47]. Similarly, anti-HIV-1 Rev aptamers can substitute for the wild-type Rev-binding element[48], indicating that sequences selected *in vitro* can function *in vivo*. When anti-Rev aptamers are expressed on their own within tissue culture cells, they act as 'decoys' for Rev and thus inhibit the replication of HIV-1 (Ref. 49).

Aptamers that target extracellular proteins have been shown to influence cell growth and differentiation, inflammation and immune function. Anti-basic fibroblast growth factor (bFGF) aptamers selected from RNA pools could inhibit binding of bFGF to cell-surface receptors at concentrations as low as 5 nM (Ref. 50), while anti-VEGF aptamers selected from RNA pools inhibited the binding of VEGF to its cognate receptor with an ED_{50} = 20–40 nM (Ref. 37). Anti-thrombin aptamers have been shown to block blood clotting in standard assays[51] and may potentially be used to prevent, transiently, the reocclusion of blood vessels following angioplasty. The anti-hNE aptamers splinted to ValP (described above[46]) have been used to inhibit inflammation in the lung[52]. In these experiments, inflammation was initiated by the intratracheal deposition of anti-bovine serum albumin (BSA) antibodies followed by an intravenous injection of BSA. When the anti-hNE aptamers were administered along with the anti-BSA antibodies they significantly decreased myeloperoxidase activity, a measure of neutrophil presence in inflamed tissue. Similarly, anti-L-selectin aptamers selected from a DNA pool blocked the *in vitro* recruitment of lymphocytes and neutrophils to epithelial cells[53]. When the anti-L-selectin aptamers were injected intravenously into severe combined immunodeficiency (SCID) mice, they prevented the localization of human lymphocytes to the peripheral and mesenteric lymph nodes. Anti-IgE aptamers selected from a DNA pool have been shown to block interactions with the high affinity IgE receptor FcεRI and inhibit IgE-mediated serotonin release from cells

in tissue culture; they might therefore prove to be useful for blocking local inflammatory responses mediated by IgE (Ref. 54). The anti-insulin receptor antibodies cited above were derived from the sera of patients suffering from an autoimmune disorder, and the anti-antibody aptamers were found to block interactions between the antibodies and the insulin receptor[44]. Similarly, aptamers selected to bind to a monoclonal autoantibody that was raised against the acetylcholine receptor could cross-react with some of the autoantibodies from patients suffering from myasthenia gravis[55].

Improving aptamer stability

The affinities and specificities of aptamers and their demonstrated abilities to inhibit protein function and modulate physiology, strongly support the use of aptamers as drugs. However, a major reservation that is frequently raised to the therapeutic application of aptamers is their relative fragility. For example, unmodified RNA molecules have been found to have half-lives of <10 min in sera[11]. Several strategies for nucleic acid stabilization have been pursued, but the most successful strategy to date has been the inclusion of modified nucleotides in selection procedures. To use modified nucleotides as part of a selection scheme, they must be able to form Watson–Crick-style base pairs and serve as substrates for enzymes such as RNA and DNA polymerases and RTs[10,56]. Several modified nucleotides, including pyrimidine nucleotides with 2′ substitutions of ribose or with adducts at the 5′ position of the base[11], meet these criteria and have been successfully used in selection experiments. For example, anti-bFGF aptamers selected from a pool that contained exclusively 2′-aminopyrimidines were 1,000-fold more stable in 90% human serum than unmodified nucleic acids[56].

Similarly, anti-hNE aptamers containing 2′-aminopyrimidines had half-lives of >20 h in serum and >9 h in urine[11], while anti-VEGF aptamers containing 2′-aminopyrimidines were stable for >17 h in urine[57]. Post-selection substitutions have also been used to increase aptamer stability; Green *et al.*[12] were able to substitute all but two of the 2′-hydroxyl groups of an anti-HIV-1 RT aptamer with 2′-methoxyl groups and yet the aptamer retained near maximal binding activity.

In general, the chemical modifications introduced into aptamers have not affected their function. For example, 2′-aminopyrimidine aptamers selected to bind the anti-insulin receptor antibody bound nearly as well as RNA aptamers and blocked patient autoantibodies, but were more than 10,000-fold more stable in sera[58]. Some modifications have even led to functional improvement. For example, the modified anti-cytokine aptamers had even greater affinities for their cognate targets than aptamers selected from RNA or DNA pools. In addition, the modified anti-bFGF aptamers could block cellular proliferation at concentrations of 50–100 nM (Ref. 56).

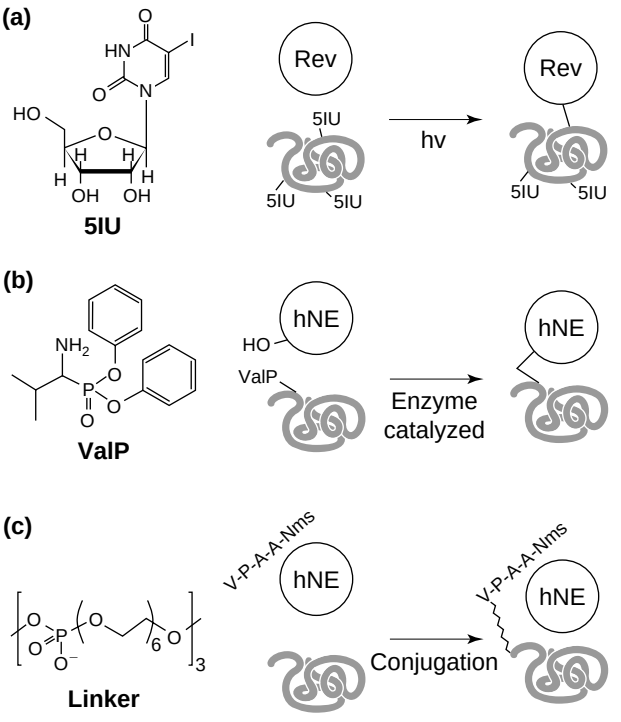

Figure 3. *Strategies for directing aptamer–target interactions. (a) Inclusion of a crosslinking reagent within a pool. When irradiated with UV light, 5-iodouridine forms covalent bonds to cysteine and aromatic residues in proteins. When incorporated into random RNA pools, 5-iodouridine allowed aptamers to be selected that could specifically crosslink to HIV-1 Rev[13]. (b) Inclusion of an inhibitor at the end of a pool. The mechanism-based inhibitor ValP was appended to a DNA pool via a non-covalently bound oligonucleotide 'splint'. The active-site serine can displace a phenol from ValP, forming a covalent linkage to the inhibitor. Anti-hNE aptamers were selected in which the ValP bound to the active site of the enzyme while the folded DNA or RNA structure bound to adjacent residues or regions[45,46]. (c) Linking aptamers and other ligands. An anti-hNE aptamer was joined to a tetrapeptide inhibitor of hNE via a flexible linker (the thioether bond between the peptide and linker is not shown). As the two compounds bind to different parts of the protein, the affinity of the conjugate is much greater than that of either of the compounds alone[14].*

Another technique that has been used to improve the stability of aptamers relies on the fact that the mirror image of a tight and specific molecular interface will itself be tight and specific. To this end, spiegelmers (from the German 'spiegel' for mirror) that contain L-enantiomers of nucleotides, rather than the natural D-enantiomers, have been generated.

To generate spiegelmers, a mirror-image target such as D-arginine[59] or L-adenosine[60], is used to select aptamers from a D-RNA pool. The L-RNA aptamers are then synthesized and these can bind to the natural target (e.g. L-arginine or D-adenosine). The L-RNA aptamers are relatively stable against nucleases, which have largely evolved to recognize D-RNA or D-DNA molecules. For example, the spiegelmers against arginine and adenosine showed no sign of degradation in human serum after 60 h of incubation at 37°C, while the corresponding D-forms were degraded within ~12 s (Ref. 60). Furthermore, an L-DNA aptamer selected to bind the peptide hormone vasopressin was shown to be stable in human serum after incubation for seven days[61]. Although these stability enhancements are impressive, this technique is currently both limited to targets that can be easily synthesized in the opposite chirality and costly in terms of synthesizing the L-form phosphoramidites.

Target validation and identification

Although the potential therapeutic applications of aptamers are exciting, most companies cannot afford to invest the time, personnel or money into the R&D that is necessary for aptamer commercialization. However, the selection methods that have been described to date can be used to augment the discovery and development of small, organic lead compounds. First, because aptamers are highly specific for their cognate targets and have been shown to generate relevant physiological effects, they can potentially be used as drug mimics for target validation. An increasing number of promising drug targets are being defined purely genetically. Aptamers selected to bind to and inhibit a given gene product could also be used to confirm or overturn hypotheses about the predicted physiological relevance of gene products. Furthermore, aptamers could be used to predict in advance whether inhibition of a target would incur serious side effects or engender the development of tolerance or resistance.

Second, aptamers can potentially be used to divine, rather than confirm, new drug targets. Although nature has generated numerous compounds (such as aminoglycoside antibiotics, netropsin and calichemycin) that act on nucleic acid targets, drug discovery efforts are generally not directed against nucleic acid targets. However, the fact that aptamers can be readily selected to bind many small organic compounds[62] conversely means that many small organic compounds can potentially recognize nucleic acid sequence and shape. Selection experiments can therefore be used to determine which sequences bind to which compounds and vice versa. For example, aptamers have been selected to bind several different aminoglycoside antibiotics[63–65].

Analysis of the selected binding species has revealed preferred sequence and structural motifs, and by comparing these features with the wealth of sequence information available in sequence databases, previously unknown correlations between compounds and targets can be generated. For example, a high affinity anti-lividomycin aptamer has been shown to be similar to sequences found in *Haemophilus influenzae* and *Leishmania* RNA molecules[66]. This roundabout identification of common sequence motifs can be bypassed by directly selecting genomic RNA or DNA molecules for their ability to bind to targets (so-called genomic SELEX)[67,68]. Although using aptamers for target identification obviously runs counter to most target-driven drug discovery efforts, such 'reverse drug discovery' techniques[69] may nonetheless prove to be useful for increasing the commercial lifetimes or diversifying the therapeutic applications of compounds that have already been approved for clinical use.

Aptamers as research reagents

Similar to antibodies, aptamers may also prove to be useful as powerful research tools for drug discovery. Again recalling the high specificity of aptamers for their targets, aptamers can potentially be used to follow proteins during purification or to localize proteins within an organism. For example, aptamers have been used as fluorescent[70] or radiolabeled[71] markers for proteins. The fluorescent aptamers have been used to quantitate protein concentrations in an ELISA-like assay (ELONA, enzyme-linked oligonucleotide assay[72]) and to follow cell-surface proteins using a fluorescence-activated cell sorter[70]. Finally, an anti-hNE aptamer splinted to ValP (Ref. 46), as discussed above, has been used to localize hNE within an organism[73]. Rats were injected intradermally with anti-BSA antibodies in one forelimb, followed by an intravenous tail injection of BSA. The accumulation of immune complexes led to neutrophil activation and inflammation. The rats were then injected with either a random DNA oligonucleotide, an IgG specific for neutrophil elastase or the anti-hNE aptamer. These compounds were all labeled with ^{99m}Tc, to allow for imaging.

The negative DNA control showed no specific retention of ^{99m}Tc. The anti-hNE IgG retained the radiolabel and was slowly cleared from circulation resulting in a high level of background signal. The anti-hNE aptamer was also specifically retained, but more quickly yielded an even higher target-to-background ratio than the antibody. Overall, these experiments suggest that aptamers may be useful for medical imaging and also may be utilized to probe the localized physiological effects of lead compounds.

Implementing nucleic acid selection

Although only a few companies will probably wish to undertake the extensive R&D efforts required to generate aptamer pharmaceuticals, nucleic acid-selection techniques are now suitably well established that they should be generally included throughout the drug discovery process. One or more researchers within a company could learn the details of nucleic acid-selection techniques and apply their unique knowledge to a range of problems; for example, aptamers could be used as relatively cheap alternatives to antibodies, either for the small- or large-scale purification of protein drugs or for following the tissue distribution of a promising lead compound. Larger companies might institute nucleic acid selection as a technical specialization, with aptamers being prepared for other divisions as readily as are oligonucleotides or monoclonal antibodies.

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