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Aptamers as Reagents for High-Throughput Screening

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ABSTRACT

The identification of new drug candidates from chemical libraries is a major component of discovery research in many pharmaceutical companies. Given the large size of many conventional and combinatorial libraries and the rapid increase in the number of possible therapeutic targets, the speed with which efficient high-throughput screening (HTS) assays can be developed can be a rate-limiting step in the discovery process. We show here that aptamers, nucleic acids that bind other molecules with high affinity, can be used as versatile reagents in competition binding HTS assays to identify and optimize small-molecule ligands to protein targets. To illustrate this application, we have used labeled aptamers to platelet-derived growth factor B-chain and wheat

germ agglutinin to screen two sets of potential small-molecule ligands. In both cases, binding affinities of all ligands tested (small molecules and aptamers) were strongly correlated with their inhibitory potencies in functional assays. The major advantages of using aptamers in HTS assays are speed of aptamer identification, high affinity of aptamers for protein targets, relatively large aptamer-protein interaction surfaces, and compatibility with various labeling/detection strategies. Aptamers may be particularly useful in HTS assays with protein targets that have no known binding partners such as orphan receptors. Since aptamers that bind to proteins are often specific and potent antagonists of protein function, the use of aptamers for target validation can be coupled with their subsequent use in HTS.

INTRODUCTION

Aptamers are structurally unique nucleic acids derived from random libraries that are capable of binding other molecules with high affinity and specificity (3,14). To date, aptamers that bind to small organic molecules, carbohydrates, amino acids, peptides, and proteins have been identified, illustrating the multitude of binding specificities present in large collections of nucleic acid sequences (6,10). With

protein targets, aptamers exhibit equilibrium dissociation constants (K_d s) typically in the nanomolar (and sometimes picomolar) range (6). Given these unique binding properties, nuclease-stabilized and appropriately formulated aptamers have substantial potential as therapeutics. For therapeutic uses, aptamers, like antibodies, are delivered parenterally (or locally) because of their limited oral availability.

In this report, we demonstrate that aptamers can be used as a screening tool to facilitate the identification of non-aptamer ligands. Drug discovery in most pharmaceutical companies is focused on the identification of orally active compounds. High-throughput screening (HTS) of appropriate libraries of compounds (generally small molecules) against validated targets constitutes one of the major activities in discovery research groups in which the overall goal is to create and/or maintain a pipeline of new drug candidates. The development of HTS assays that allow efficient detection of active compounds from such libraries is a central component of drug discovery. Here, we show that aptamers can be used in competition binding assays to identify compounds that are capable of displacing the aptamers from their targets. The affinities of competitor compounds can

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

be calculated from the known affinity of the aptamer for its target and the competition binding profiles. The method is highly versatile and compatible with a variety of HTS platforms because aptamers, as chemically synthesized molecules, can be labeled with various functional groups without compromising their binding properties. We illustrate the method with two protein targets: platelet-derived growth factor (PDGF) and wheat germ agglutinin (WGA). For each protein, a small focused set of molecules is screened in a competition binding assay: low molecular weight organic anions for PDGF and oligosaccharides for WGA. We show that, for both PDGF and WGA, the best ligands can readily be identified and that binding affinities of the competitors correlate strongly with their activities in *in vitro* assays.

MATERIALS AND METHODS

Materials

Human recombinant PDGF-BB expressed in *E. coli* was purchased from R&D Systems (Minneapolis, MN, USA) as a carrier-free lyophilized powder. WGA (*Triticum vulgare*) was purchased from EY Laboratories (San Mateo, CA, USA).

Oligonucleotides were synthesized in our laboratories using a model 394 oligonucleotide synthesizer (Applied Biosystems, Foster City, CA, USA) according to optimized protocols. PDGF aptamer 20ta is a synthetic 33-mer DNA oligonucleotide of sequence 5'-CGGGCGCGTTCTTCGTGGTACT-TTTAGTCCCG-3', aptamer 20tb is a synthetic 27-mer DNA oligonucleotide of sequence 5'-GGGCCGTTTCGGGTTACTTTTAGTCCC-3', and aptamer PD316 is a synthetic oligonucleotide containing some modified (2'F and 2'O-methyl, italic and bold letters, respectively) bases, and 18-atom PEG spacers replacing some bases, to increase serum stability, of sequence 5'-T_{NH2}CAGGCUACG[spacer]CGTAGAGCAUCA[spacer]TGATCCUG-3'3'T-3' (4). WGA aptamer 11.20 is a 98-mer 2'-aminopyrimidine RNA prepared by *in vitro* transcription (1).

Organic anions used in the PDGF competition assay were Evans blue, trypan blue, amaranth, sulfonazo III, New Coccine, *myo*-inositol hexasulfate, (2-(4-sulfophenylazo)-1,8-dihydroxy-3,6-naphthalene disulfonic acid (SPADNS), calcion, and azocarmine B from Sigma-Aldrich (Milwaukee, WI, USA); naphthalene 1,3,6-trisulfonic acid (NTSA) from Fluka (Ronkonkoma, NY, USA), suramin from Calbiochem-Novabiochem (San Diego, CA, USA), and sucrose octasulfate from Toronto Research Chemicals (Toronto, Canada).

Oligosaccharides used in the WGA aptamer competition assay were: N-acetyl-D-glucosamine (GlcNAc) from Sigma (St. Louis, MO, USA); N,N-di-acetylchitobiose (GlcNAc)₂, N,N,N'-triacylchitotriose (GlcNAc)₃, N,N',N'',N'''-tetraacyltetraose (GlcNAc)₄, galactose1-β-3[fucose-α-4]glucosamine (Lewis A trisaccharide), galactose1-β-4[fucose 1-α-3]glucosamine (Lewis X trisaccharide), fucose1-α-2 galactose1-β-4[fucose 1-α-3]glucosamine (Lewis Y tetrasaccharide), Sialyl Lewis A, Sialyl Lewis X, fucose1-α-4glucosamine, fucose1-α-3glucosamine, N-acetyl-lactosamine (LacNAc), 3'α-Sialyl-N-acetylglucosamine (α-Sialyl-LacNAc), 3'β-Sialyl-N-acetylglucosamine (β-Sialyl-LacNAc) from Toronto Research Chemicals.

Small-Molecule/Aptamer Competition Assays

Organic anions and oligosaccharides were weighed and dissolved in binding buffer (HBSMC: 25 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1 mM CaCl, pH 7.4) to prepare stock solutions. For organic anions in the naphthalene sulfonic acid class, stock solution concentrations were corrected for the purity stated by the manufacturer. Oligonucleotide concentrations were determined from the absorbance at 260 nm and extinction coefficients of 33 μg/mL/a.u. for DNA aptamers (20ta and 20tb) and 37 μg/mL/a.u. for modified aptamers (PD316, NX1838-sp, and 11.20).

In the PDGF assay, the competitors at varying concentrations were mixed with the 5'-³²P end-labeled 20tb ligand at room temperature. PDGF was then added to the mixture and allowed to

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

equilibrate for 45 min at room temperature, followed by 15 min at 37°C. All competition mixtures (90 μ L volume) contained 5'- 32 P end-labeled 20tb at 1.2 nM, PDGF-BB at 1.0 nM in HBSMC containing 0.0067% human serum albumin (HSA). Aptamer bound to PDGF-BB was separated from free aptamer by filtration through pre-washed 0.45- μ m nitrocellulose membrane filters (Millipore, Bedford, MA, USA), followed by washing of the filters with 5 mL HBSMC at room temperature. The fraction of 32 P end-labeled 20tb bound at each competitor concentration was determined by scintillation counting.

In the WGA assay, the oligosaccharides (or cold 11.20) competitors at varying concentrations were mixed with the 5'- 32 P end-labeled 11.20 ligand at room temperature. WGA was then added to the mixture and allowed to equilibrate for 60 min at room temperature. All competition mixtures (90 μ L volume) had 32 P end-labeled 11.20 at 10 nM, WGA at 10 nM in HBSMC containing 0.0067% HSA. Filter binding was done as described above.

Analysis of Competition Data

Competition experiments were analyzed according to a model in which two molecules (R and C) bind to the same target (P) in a mutually exclusive manner with dissociation constants K_{dR} and K_{dC} respectively, assuming 1:1 stoichiometry for both competitor species (1). With defined values for K_{dR} and $[P]_{total}$, the competition data were fit to the corresponding competition equation (1) to obtain least squares values for K_{dC} . In these curve fits, the midpoint of the competition does not necessarily correspond to the K_{dC} .

PDGF-BB-Stimulated [3 H]-Thymidine Incorporation Assay

A10 rat smooth muscle cells were plated in 96-well tissue culture plates at 7.5×10^3 /well in 100 μ L DME + 4.5 g/L glucose + HEPES + 0.1% FBS and starved for about 20 h. Aptamers or small-molecule organic anions at varying concentrations were added to cells followed by PDGF-BB at 10 ng/mL. After 6 h at 37°C in a 5% CO₂ atmos-

Table 1. Structures of Small-Molecule Competitors Used in the PDGF Competition Assay

Competitor	Structure	K_{dC} , μ M	K_{dR} , μ M
Evans Blue		0.15 ± 0.04	0.10 ± 0.04
Trypan Blue		0.26 ± 0.06	0.12 ± 0.04
Suramin		1.1 ± 0.4	1.3 ± 0.07
Calcion		7.8 ± 1.2	4.3 ± 1.1
SPADNS		19 ± 6.0	49 ± 15
Azocarmine B		22 ± 3.0	18 ± 7.1

phere, [3 H]-thymidine was added (0.25 μ Ci/well) and incubated for another 24 h. Cells were then lysed in 1% Triton® X-100 for 20–30 min on a slow shaker at room temperature, harvested onto 96-well glass fiber filter plates, and dried. The amount of incorporated [3 H]-thymidine radioactivity was determined by scintillation counting. K_i values for inhibition of PDGF-BB-induced [3 H]-thymidine uptake were obtained by fitting the data to the same equations used in the competition binding analyses (1).

Agglutination Assay

WGA-induced agglutination of sheep erythrocytes was performed as de-

scribed by Bridonneau et al. (1), with the exception of substituting gelatin veronal buffer with HBSMC + 0.0067% HSA buffer in the agglutination reaction mixture. Briefly, agglutination induced by WGA prevents readily observable settling of erythrocytes to the bottom of the round-bottom wells in 96-well plates. Sheep blood in Alsever's buffer (Sierra for Medical Science) was pelleted and washed in HBS + 10 mM EDTA, then resuspended at 10% red blood cells (RBCs) (v/v) in HBS. Just before use, the cells were diluted to 1% (3×10^8 cells/mL) in HBSMC. Oligosaccharide or aptamer inhibitors at varying concentrations were mixed with WGA at room temperature in a Falcon® 96-well round-bottom microplate (Becton Dickinson,

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

Table 1. Continued

Competitor	Structure	$K_{DC}, \mu M$	$K_i, \mu M$
Amaranth		48 ± 16	26 ± 3.9
Sulfonazo III		74 ± 27	40 ± 19
New Cocaine		120 ± 20	170 ± 68
myoinositol hexasulfate		720 ± 200	150 ± 84
Naphthalene 1,3,6-trisulfonic acid		870 ± 400	>1000
Sucrose octasulfate		1700 ± 450	44 ± 7.2
PDGF Aptamer (20ta)		0.000050 ± 0.000055	
PDGF Aptamer (20tb)		0.00039 ± 0.00016	
PDGF Aptamer(PD 316)		0.000070 ± 0.000030	0.0000021 ± 0.00000084
VEGF Aptamer (NX1838sp)		4.9 ± 2.2	0.35 ± 0.28

K_{DC} values were calculated from the competition profiles and K_i values derived from $[^3H]$ -thymidine incorporation assays (Figure 1). Dashed lines indicate a 2-fold (c_{2v}) axis of symmetry. K_{DC} and K_i values of PDGF aptamers 20ta, 20tb, PD316, and VEGF aptamer NX1838sp are included for comparison.

Franklin Lakes, NJ, USA), then sheep erythrocytes were added and incubated at room temperature for 2–3 h. Agglutination in each well was scored as “complete”, “partial”, or “absent”. Assay mixtures (120 μ L volume/well) contained 40 nM WGA and sheep erythrocytes at 1×10^8 cells/mL. The effect of various test compounds (oligosaccharides and aptamers) in 2-fold dilutions was evaluated in the absence and presence of WGA. The IC_{50} values were estimated by visual inspection of the plates. The effect of various test compounds (oligosaccharides and aptamers) in 2-fold dilutions was evaluated in the absence and presence of WGA. The IC_{50} values were estimated by visual inspection of the plates.

RESULTS

Screening of Organic Anions that Bind to PDGF-BB

For the PDGF competition assay, we used a DNA aptamer identified previously (8). In the SELEX experiment that yielded this aptamer, we aimed simply to identify aptamers with the highest affinity for PDGF-AB, and no special measures were taken to direct aptamer binding to a particular site on the protein (8). All aptamers obtained in this SELEX experiment bound preferentially to the B-chain of PDGF (although both PDGF A-chain and B-chain were present in equal molar ratio, as covalently linked heterodimer, in all affinity selections). Representative aptamers inhibited the binding of PDGF-BB to cell surface receptors and PDGF-BB-induced DNA synthesis in vitro. Using photo-cross-linking experiments, we have shown that a specific nucleotide in the aptamer interacts with phenylalanine-84 of the PDGF B-chain located near the region of PDGF known to be involved in receptor binding (8).

Most aptamers in the affinity-enriched pool bound to PDGF B-chain with very high affinities (K_d values of about 100 pM or lower) (8). In the competition assay, we aimed to identify compounds that bind to PDGF-BB with K_d values in the micromolar to millimolar range to approximate typical affinities of first-generation leads in HTS as-

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

says. Given this arbitrary affinity range and the constraints imposed by the competition equations (1), computational modeling studies have shown that the affinity of a truncated aptamer 20ta ($K_d = 60$ pM) was considerably higher than optimal. The major limitation here was the requirement for above millimolar concentrations of competitors to achieve aptamer displacement. Aside from reagent cost and availability issues (that in this case were not limiting), such high organic anion concentrations would require an adjustment of NaCl concentration in the binding buffer to maintain constant ionic strength. We therefore reduced the affinity of aptamer 20ta (33-mer) about 10-fold by additional truncations to yield aptamer 20tb (27-mer, $K_d = 0.65$ nM).

We chose to screen a panel of organic anions (mostly in the naphthalene sulfonic acid class) for their ability to displace a 32 P-radiolabeled aptamer from PDGF-BB. These compounds were chosen because suramin and several other members of this family inhibit the binding of PDGF to cells that express the PDGF receptors (5,11). Since the PDGF aptamer also inhibits receptor binding (and with considerably greater potency), it was reasonable to expect that suramin and the aptamer bind to PDGF in a mutually exclusive manner. We used the nitrocellulose filter binding method to separate bound from unbound aptamer. Structures of competitors are shown in Table 1 along with the corresponding K_{dC} values (K_d values calculated from the competition profiles; Figure 1). For all competition experiments, the concentrations of the 32 P-labeled 20tb aptamer and PDGF-BB were 1.2 nM and 1.0 nM, respectively. Binding reactions were done at 37°C, and the time of incubations was at least 60 min to ensure that equilibrium was established ($t_{1/2}$ values of dissociation for similar aptamers from PDGF-AB are 2–7 min) (8). Fast dissociation rates are clearly advantageous in these assays because they reduce the length of time required to reach equilibrium.

Among the 12 organic anions examined, there is a wide range of affinities for PDGF-BB (Table 1). Inspection of the structures in this focused library suggests that the placement of sulfonate or sulfate groups in space (or anions in gen-

Figure 1. PDGF-BB assays: competition binding (left-hand panels) and inhibition of [3 H]-thymidine incorporation (right-hand panels) by aptamers and organic anions.

Figure 2. Double logarithmic plot of K_{dC} (binding to PDGF-BB) versus K_i (inhibition of PDGF-BB-induced [3 H]-thymidine incorporation) values for aptamers and organic anions (from Table 1 and Figure 1). The line corresponds to the least squares fit of the $\log K_i$ versus $\log K_{dC}$ plot (slope = 1.08 ± 0.9 , correlation coefficient = 0.961).

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

Table 2. Structures of Oligosaccharide Competitors Used in the WGA Competition Assay

Competitor	Structure	K_{dC} , μM	IC_{50} , μM
GlcNAc		13000 ± 6000	3500
(GlcNAc) ₂		83 ± 21	94
(GlcNAc) ₃		4 ± 0.3	16
(GlcNAc) ₄		1.3 ± 0.3	8
(GlcNAc) ₅		1.0 ± 0.2	6
(GlcNAc) ₆		0.71 ± 0.01	4
Fuc1- α -4GlcNAc		8100 ± 5200	>6000
Fuc1- α -3GlcNAc		6600 ± 5300	>6000

eral) is more important for binding than the overall negative charge. For example, suramin, a hexaanion, is not the best ligand for PDGF-BB. Evans blue and trypan blue, both tetraanions, bind to PDGF with 4- to 7-fold higher affinity than suramin. Conversely, *myo*-inositol hexasulfate and sucrose octasulfate (a hexaanion and an octaanion, respectively) have comparatively poor affinities for PDGF-BB. In addition, the affinities of structurally related SPADNS and NTSA, both trianions, differ by about 50-fold. Although there are undoubtedly important factors that influence binding other than charge placement, it is clear that at least some conclusions about structure-activity relationship can be obtained even from a relatively small focused library such as the one shown in Table 1.

Effect of Organic Anions on PDGF-BB-Induced [³H]-Thymidine Incorporation

We next tested the same panel of molecules for their ability to inhibit PDGF-BB-induced [³H]-thymidine incorporation in A10 rat smooth muscle cells. Following induction of quiescence by incubation in serum-free media, the A10 cells were incubated with 10 ng/mL PDGF-BB for 30 h in the presence of varying concentrations of competitors. The amount of [³H]-thymidine incorporated into DNA at the end of 30 h was determined by scintillation counting and normalized to the level of incorporation observed in the absence of competitors (Figure 1). The K_i values (Table 1) were obtained by

fitting the data points to the competition equation (1), assuming a receptor concentration of 10^{-12} M (7500 cells/100- μL well, 10^4 PDGF receptors/cell) and a K_d of 10^{-11} M for the binding of PDGF-BB to PDGF receptors (12). For the purposes of curve fitting, the K_i values are analogous to the K_{dC} values in the above competition binding assay.

A double logarithmic plot of K_{dC} versus K_i values (Figure 2) demonstrates a strong correlation between binding affinity and inhibitory potency in vitro for all compounds tested (slope of the least squares line = 1.08 ± 0.09 , correlation coefficient = 0.961). Figure 2 includes the points for the PDGF aptamer (PD316, modified for nuclease resistance required for the in vitro assay) (4) and the control VEGF aptamer (NX1838sp) (13). As expected, the PDGF aptamer is both the best ligand and the most potent inhibitor. For comparison, the VEGF aptamer NX1838sp is a weaker ligand and a less potent inhibitor by five and four orders of magnitude, respectively. The fact that several organic anions are better ligands and more potent inhibitors compared to the VEGF aptamer (a 28-mer oligonucleotide) is further evidence that negative charge alone is not sufficient to account for the differences in binding affinity to PDGF-BB.

Screening of Oligosaccharides that Bind to WGA

For the WGA competition assay, we used a 2'-aminopyrimidine RNA aptamer 11.20 identified previously (1). Aptamer 11.20 was selected by incubating a randomized nucleic acid library with WGA immobilized on solid support, removing the unbound molecules by extensive washing, and then displacing the aptamers bound to a specific site on WGA with a competitor, (GlcNAc)₃. Thus, in contrast to the PDGF SELEX described above in which high-affinity binding was the only selection pressure, the WGA aptamers were selected for binding to a specific site on the protein. Not surprisingly, 11.20 and related aptamers could be displaced with (GlcNAc)₃ (1). In addition, these aptamers potently inhibited WGA-mediated agglutination of sheep erythrocytes.

We screened a group of oligosaccha-

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

rides that contain the GlcNAc group for their ability to displace radiolabeled 11.20 from WGA using the nitrocellulose filter binding method (Table 2). Among these compounds, we observed a wide range of affinities for WGA. Excluding the aptamer, as previously recognized (7), the best ligands were multimers of GlcNAc, with only modest additional improvement in binding affinity observed with multimers larger than (GlcNAc)₃ (Table 2 and Figure 3). The monomer GlcNAc at concentrations up to 10 mM was unable to appreciably displace the aptamer from WGA. Among the other compounds tested, LacNAc (Gal1- β -4GlcNAc) and its derivatives sialylated at the 3-position of galactose showed improved binding compared with GlcNAc alone. The remaining oligosaccharides tested bound to WGA with affinities similar to that of the GlcNAc monomer.

Effect of Oligosaccharides in the Agglutination Assay

We tested the same oligosaccharides for their ability to inhibit WGA-mediated agglutination of sheep erythrocytes. In this assay, sheep erythrocytes were incubated in buffer containing tested oligosaccharides or aptamers at varying concentrations in the presence or absence of WGA. Agglutination prevents settling of erythrocytes to the bottom of the round-bottom wells, a process readily visible to the naked eye (Figure 3B). GlcNAc and its multimers inhibited WGA-induced agglutination with IC₅₀ values that closely matched their binding affinities for WGA. The evaluation of WGA agglutination inhibitory activity for some oligosaccharides was complicated by their ability to *induce* agglutination in the absence of WGA. This was observed with (GlcNAc)₄ (EC₅₀ = 250 μ M) and (GlcNAc)₆ (EC₅₀ = 1 mM) (Figure 3B, upper plate), as well as with Lewis X (EC₅₀ = 1 mM), Lewis A, Lewis Y, Sialyl Lewis A, Sialyl Lewis X, and Sialyl- β 3-LacNAc (EC₅₀ = 125 μ M) (data not shown). For (GlcNAc)₄ and (GlcNAc)₆, this did not interfere with the determination of the IC₅₀ value for inhibition of WGA-induced agglutination (Figure 3). However, in the case of Lewis A, Lewis X, Lewis Y, Sialyl Lewis A, Sialyl Lewis X, and Sialyl- β 3-LacNAc, this did not interfere with the determination of the IC₅₀ value for inhibition of WGA-induced agglutination (Figure 3). However, in the case of Lewis A, Lewis X, Lewis Y, Sialyl Lewis A, Sialyl Lewis X, and Sialyl- β 3-LacNAc, this did not interfere with the determination of the IC₅₀ value for inhibition of WGA-induced agglutination (Figure 3).

Table 2. Continued.

Competitor	Structure	K _d , μ M	IC ₅₀ , μ M
Lewis A		1500 $\pm$ 400	(>125)
Lewis X		2600 $\pm$ 1400	(>1000)
Lewis Y		5400 $\pm$ 7000	(>125)
Sialyl Lewis A		6100 $\pm$ 1000	(>125)
Sialyl Lewis X		2800 $\pm$ 300	(>125)
LacNAc		480 $\pm$ 250	4500
Sialyl- α -3LacNAc		540 $\pm$ 170	>1000
Sialyl- β -3LacNAc		440 $\pm$ 200	(>125)
WGA Aptamer (11.20)		0.0057 $\pm$ 0.0009	0.19
VEGF Aptamer (NX1838sp)		16 $\pm$ 4.5	>32

K_d values were calculated from the competition profiles, and IC₅₀ values were derived from the agglutination assays (Figure 3). K_d and IC₅₀ values of aptamers 11.20 and NX1838sp are included for comparison. For oligosaccharides in which WGA-independent agglutination interfered with the assay, the minimal IC₅₀ values are given in parentheses.

LacNAc, the EC_{50} values for WGA-independent agglutination were below the expected inhibitory concentration for WGA-induced agglutination. Therefore, these IC_{50} values could not be determined. Compared to the oligosaccharides, the aptamer (11.20) was a considerably more potent inhibitor (Table 2). Several oligosaccharides were better inhibitors than the irrelevant aptamer (NX1838sp).

For WGA, as with PDGF-BB, a double logarithmic plot of K_{IC} versus IC_{50} values (Figure 4) demonstrates a strong correlation between binding affinity and inhibitory potency (slope of the least squares line = 0.74 ± 0.05 , correlation coefficient = 0.978).

DISCUSSION

We have shown here that aptamers can be used in HTS assays to identify active compounds from libraries of small molecules. Our work complements the recent report of Hyde-DeRuyscher et al. (9) in which peptides derived from phage-displayed combinatorial libraries were used in an analogous manner. In drug discovery, HTS assays often require protein-binding reagents whose displacement by various test compounds is indicative of potential biological activity of those compounds. In assays that screen for compounds that disrupt protein-protein, protein-nucleic acid, or enzyme-substrate interactions, the use of natural ligands or substrates (or substrate analogs) is advantageous because the correlation between displacing ability and functional inhibition is by necessity high. However, natural binding partners are often not readily available, difficult to produce in sufficient quantities, difficult to label, or not known (e.g., orphan receptors). In such cases, the use of aptamers can be advantageous in several ways.

First, aptamer identification is generally rapid, requiring 3–15 rounds of SELEX to generate affinity-enriched pools that bind to protein targets with affinities in the nanomolar to picomolar range. A round of SELEX using manual protocols takes about one day and considerably less than that using automated protocols. Since SELEX with

immobilized protein targets is common, performing simultaneous SELEX experiments in an automated format is straightforward. Subsequent cloning and sequencing steps that generally take several weeks can also be automated. It should be noted that for use in HTS competition assays, the affinity of aptamers for their targets need not be very high because the affinity of first-generation lead compounds will typically be considerably lower than that of the aptamer. For example, we found it useful to reduce the affinity of the PDGF aptamer about 10-fold to facilitate the identification of competitors that bind PDGF with micromolar affinities. Therefore, the identification of aptamers with affinities adequate for HTS should often require fewer SELEX rounds (and thus less time). As illustrated with the WGA example (1), aptamers directed to specific sites on proteins can also be selected, provided that a known ligand is available.

Second, the fact that aptamers are generally synthesized chemically will facilitate the conversion of rather cumbersome bench-top assays (such as ours) into real HTS assays. Screening of even modest libraries (e.g., 10 000 compounds) would require the use of 96-, 384-, or 1536-well microplates. In one version of such an assay, the protein targets can be immobilized on the surface of microplates (by hydrophobic adsorption, biotin-avidin coupling, or chemical coupling), followed by the addition of labeled aptamer in the presence or absence of compounds to be screened. Since aptamers typically bind to their targets with rapid association and dissociation rates (1,8), incubation times of less than 60 min will usually be sufficient. Following washing, the amount of aptamer bound to the immobilized protein can be determined by automated plate reading. As chemically synthesized molecules, aptamers are amenable to a variety of labeling procedures. In our assays, we have used ^{32}P labeling because of ease of detection and high sensitivity. Radioactivity is still frequently used in HTS laboratories; however, the inherent hazard, expense, and inconvenience provide an impetus to use other detection methods. With the availability of fluorescent phosphoramidites and biotin phospho-

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

ramidites from several suppliers, aptamers can be labeled with fluorophores or biotin in a site-specific manner. Importantly, the addition of fluorophores or biotin generally does not disrupt the exquisite binding properties of aptamers. For example, fluorescein-labeled aptamer to vascular endothelial growth factor (VEGF) was recently used to detect surface-immobilized VEGF in a microplate format (2) (in

this example, anti-fluorescein antibody coupled to alkaline phosphatase was used to detect the bound aptamer using a chemoluminescence detection system). Regarding the amount of aptamer required for HTS assays, we used about 10 ng aptamer per assay in our unoptimized screens. The modest amount of aptamer required is a consequence of high-affinity binding of aptamers and their relatively small size (high-affinity binding of aptamers for protein targets is typically encoded in sequences of 20–40 nucleotides; with molecular weights of 7–14 kDa, aptamers are 10–20 times smaller than antibodies). For screening 10 000 compounds, 0.1 mg of the aptamer would be needed (assuming comparable aptamer requirements per assay). In addition, batch-to-batch variability with synthetic aptamers can be carefully controlled.

Third, the frequency of false-negative and false-positive results in competitive displacement HTS assays with aptamers should be relatively low. Since aptamers are not natural ligands for most protein targets, false-negative results will be obtained for compounds that bind to the biologically relevant site on the protein but do not displace

the aptamer. Although we observed good correlation between binding affinity and inhibitory potency with compounds tested in the PDGF and WGA assays, deviations are likely to be encountered when larger and less biased libraries are screened. To decrease the frequency of such false-negative results, one can use more than one aptamer for the screens. However, it is important to note in this context that, in most cases, aptamers identified to protein targets generally compete with each other, even when they belong to different sequence families or have different compositions (RNA, DNA, or modified RNA). In other words, aside from the formal possibility of mutually exclusive binding through non-overlapping sites, there appear to be preferred sites on proteins for aptamer binding. Nevertheless, given their size and what is known about other nucleic acid-protein interactions (15), most aptamers are likely to cover a significant surface fraction of their protein targets. This in turn should reduce the problem of false negatives in competition assays. At the same time, the opposite problem of identifying binding competitors that subsequently turn out to be inactive in

Figure 3. WGA assays. Panel A, competition binding by aptamers and oligosaccharides. Panel B, effect of representative oligosaccharides on agglutination of sheep erythrocytes without WGA (upper plate) or with 40 nM WGA (lower plate). Oligosaccharide concentrations are 2-fold dilutions across columns 1–11, from 9 mM in column 1 to 8.8 μ M in column 11 for GlcNAc, from 3 mM in column 1 to 1.5 μ M in column 11 for (GlcNAc)₂, and from 1 mM in column 1 to 0.98 μ M in column 11 for (GlcNAc)_{3–6}. Column 12 is without added oligosaccharides.

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

Figure 4. Double logarithmic plot of K_{dC} (binding to WGA) versus IC_{50} (inhibition of WGA-induced agglutination of sheep erythrocytes) values for aptamers and oligosaccharides (from Table 2 and Figure 3). The line corresponds to the least squares fit of the $\log K_{dC}$ versus $\log IC_{50}$ plot (slope = 0.74 ± 0.05 , $cc = 0.978$).

functional assays (false positives) should increase with larger surface area of aptamer-protein interaction. However, since displacing high-affinity aptamers requires a certain minimal threshold affinity of the competitors (as seen in our examples), this problem will be limited to a smaller subset of best ligands among the compounds tested. It should be noted that false-positive results would also be obtained if test compounds prevented the protein-aptamer interaction by binding to the aptamer.

Finally, with the increasing number of potential new therapeutic targets arising from genome sequencing programs, the use of aptamers for target validation can be used in conjunction with their use in subsequent HTS assays. High-affinity interaction of aptamers with their protein targets often translates to inhibition of protein function (as illustrated with PDGF and WGA aptamers). Aptamers can thus be used together with or in place of peptides, antibodies, antisense/ribozyme agents, and gene deletion/overexpression studies for testing the biological function of newly identified genes. Aptamers that inhibit the function of a protein in a target validation assay must bind in the vicinity of a functionally relevant site on the protein. The use of such aptamers in HTS assays should increase the likelihood of

identifying active compounds from libraries of drug candidates. In some cases, as with the examples shown here, none of the compounds tested will exceed the binding affinity and inhibitory potency of the aptamer. The question of whether or not it makes sense to develop the aptamer as a drug will often be asked, and the answer will depend on the comparison of usual parameters such as efficacy, bioavailability, toxicity, and cost between the aptamer and the next best drug candidate.

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