Aptamers as tools for target prioritization and lead identification

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The increasing number of potential drug target candidates has driven the development of novel technologies designed to identify functionally important targets and enhance the subsequent lead discovery process. Highly specific synthetic nucleic acid ligands – also known as aptamers – offer a new exciting route in the drug discovery process by linking target validation directly with HTS. Recently, aptamers have proven to be valuable tools for modulating the function of endogenous cellular proteins in their natural environment. A set of technologies has been developed to use these sophisticated ligands for the validation of potential drug targets in disease models. Moreover, aptamers that are specific antagonists of protein function can act as substitute interaction partners in HTS assays to facilitate the identification of small-molecule lead compounds.

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▼ Genome sequencing and large-scale proteomic projects have generated a wealth of information about different genomes and the expression status of mRNAs and proteins at various stages and states of development and disease. This explosive growth in the volume of data continually delivers new challenges for pharmaceutical R&D. As a consequence, bottlenecks in the value chain have shifted away from upstream data generation and towards downstream data processing, that is, target validation and lead identification.

Clearly, the challenge in the post-genomic era will be to develop novel strategies for prioritizing potential drug targets destined for subsequent development programs, because the effort of identifying lead compounds against the overwhelming number of often poorly validated, genomics-derived targets vastly exceeds today's R&D resources. Further along the pipeline, a new set of techniques need to be developed that can accelerate the identification and validation of pharmaceutical lead compounds, especially if they are not directed against well-characterized target families. Linking these approaches in an integrated discovery program will facilitate the rapid, parallel procession of a high number of potential drug target candidates, from target validation to lead identification.

A key step in the drug discovery process is determining the relevance of a given gene product to a particular cellular process or disease state. Traditionally, the process of target validation relies on the alteration of genomic information, either at the DNA level using gene-knockout strategies [1], or at mRNA level using antisense or RNA interference (RNAi) approaches [2–5]. One advantage of the conventional gene-knockout strategy is its power to investigate the function of a given gene in the whole mammalian organism. However, the process itself is laborious and time-consuming. By contrast, gene-knockdown methods, such as antisense or RNAi, provide a time- and cost-effective means of elucidating the functional role of a gene product. However, it is becoming increasingly clear that genomic-level technologies only give an incomplete picture of the role of a protein in a given cellular environment. For example, they eliminate all functions of the targeted gene product, thus making it difficult to simulate the effects of a small-molecule drug, which often influences specific functions of individual protein domains. Moreover, alternative splicing, post-translational modifications and multiple aspects of the four-dimensional organization of the proteome make it extremely difficult to discern which form or status of a protein encoded by a single gene has pharmaceutical relevance. That most proteins act in the context of multiprotein complexes further complicates the problem of interpreting genetic data. In yeast, a large-scale analysis of protein complexes revealed that 70% of the proteins analyzed were part of a protein complex, with

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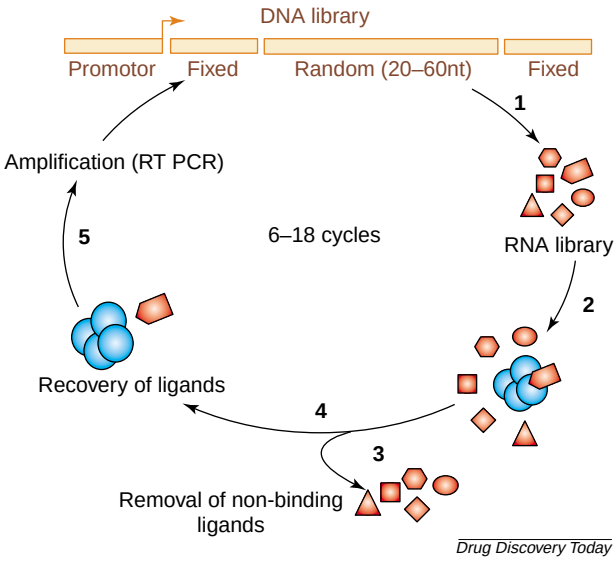

Figure 1. The *in vitro* selection process comprises five iterative steps: (1) generation of an RNA library; (2) incubation of the library with target molecule; (3) removal of non-binding sequences; (4) recovery of bound RNA; and (5) amplification of binding molecules by reverse transcription (RT)-PCR and *in vitro* transcription. This process is repeated 5–20 times and the final enriched library is cloned and sequenced to identify monoclonal RNA aptamers.

an average of twelve proteins per complex. The removal of one protein, for example, by antisense techniques, will therefore probably affect the whole complex and, in some cases, cause pleiotropic effects. These effects will complicate phenotypic predictions if the target is a good choice for a direct inhibitory approach. Finally, the scope of genomic strategies ends after the validation step, leaving the researcher with the next hurdle – developing appropriate assays to identify the first hits.

Clearly, there is a huge demand for inhibitors or modulators that are directed against a specific function of a protein, and that facilitate analysis of the protein within the context of its natural expression status. Subsequently, modulators directed to key functional sites on the target protein can be used as substitute ligands in HTS assays for the identification of pharmaceutical lead compounds, thus further simplifying the drug discovery process. Protein-based methods have traditionally been used for this purpose. However, functional nucleic acids that can bind cellular proteins or protein domains – also known as aptamers – offer a convenient and time-saving approach to understanding the biological role of the numerous and, to-date, poorly characterized target candidates resulting from genome-wide sequencing projects. As single-stranded nucleic acid molecules can fold into discrete 3D shapes [6], they are capable of binding their cognate targets with affinities and specificities comparable to, or even excelling, those of antibodies. Their characteristic features make them exceptionally well-suited for elucidating the function of a growing number of new target candidates within the context of living cells. Moreover, aptamers can also be used as substitute ligands for competitive HTS assays, circumventing the need for previous knowledge of structural, enzymatic or ligand-binding properties.

Aptamer technology: an overview

Aptamers can be routinely isolated from combinatorial oligonucleotide libraries using *in vitro* selection methods [7,8]. An *in vitro* selection experiment comprises various sequential steps, starting with the generation of an oligonucleotide library of random sequences. This library is then subjected to an appropriate selection strategy, which promotes the separation of functional molecules from non-functional molecules. The small proportion of nucleic acids with the desired activity is then enzymatically amplified (replicated) and subjected to further rounds of selection and amplification. This is necessary because the complexity of the library, which can contain up to 10^{15} different oligonucleotide sequences, makes it impossible to enrich for the active sequences in one single selection-and-amplification cycle. Repetitive selection-and-amplification rounds are performed until the functional sequences dominate the population (Fig. 1).

Since their first appearance in the literature in 1990, aptamers have been isolated against a multitude of targets ranging from small organic molecules to peptides and proteins and even complex multimeric structures [9,10]. These aptamers have shown exceptional affinity and specificity for their targets. In some cases, picomolar or even sub-picomolar affinities were obtained [11–13]. The aptamer technology has established itself as a useful tool in molecular biology and is now being evaluated for diagnostic and therapeutic procedures. Several reviews have been published that give a comprehensive coverage of the more technical aspects of aptamer selection, as well as their use in diagnostic and therapeutic applications [14–18]. The following sections focus instead on the recent technical advancements in aptamer systems as tools for target validation and small-molecule drug discovery.

High-throughput aptamer generation

Aptamers need to be generated in a way that is both simple and efficient to take full advantage of their potential as tools for prioritizing the multitude of potential drug target candidates and the subsequent process of lead identification. Generally, specific aptamers are first isolated after

6–18 cycles of repeated selection-and-amplification, which can take weeks or even months to complete when performed manually. However, there has been considerable progress over the past few years in the automation of *in vitro* selection techniques to increase the throughput of aptamer generation and evaluation. In 1998, Ellington and colleagues performed a model selection for poly(T) binding RNA molecules on a Biomek 2000 workstation (Beckman Coulter; http://www.beckman.com), thus providing the proof-of-principle for automated aptamer selection [19]. Analysis of the enriched library revealed species containing long stretches of A nucleotides, indicating a successful automated *in vitro* selection. Further work on fully automated selections against a protein target was published by the same group in 2001 [20], while Drolet *et al.* demonstrated that *in vitro* selection protocols can be adapted to parallel formats in 96-well microtiter plates [21]. Successfully implementing automated selection protocols involves two key steps: (1) effective separation of specifically binding oligonucleotide molecules from the non-binding background; and (2) efficient recovery of the target-bound species. Drolet *et al.* solved this problem by hydrophobic adsorption of the target proteins to 96-well microtiter plates, while Ellington's group used a bead-on-filter wash procedure. Furthermore, amplification methods have to be optimized for automated selection protocols to avoid the purification steps (e.g. gel electrophoresis) of reaction products, which are time-consuming and difficult to automate. However, the results justify the work involved. Using the automated *in vitro* selection platform set up at NascaCell (http://www.nascacell.de), selections against eight different targets in parallel can be performed within three days.

Aptamers for target validation

Although nucleic acids themselves have poor pharmacological properties, aptamers have been found to be effective tools for investigating the function of endogenous cellular proteins, both in extracellular and intracellular environments. Several examples have shown that aptamers can be generated against a broad range of target classes, such as kinases, phosphatases, proteases, growth factors, receptors or coagulation factors. Moreover, experimental data suggest that aptamers preferentially target sites of biological activity on a given protein, thus effectively inhibiting its function [22]. Even in cases where standard selection procedures do not result in an aptamer for a specific protein domain owing to the selected aptamers being directed to a more dominant site on the protein, it is still possible to identify an aptamer with the desired activity through special selection procedures. For example, aptamers with unwanted functions can be removed using subtractive selection protocols [23]. Besides being isolated against a multitude of different target proteins, aptamers have also shown their broad applicability through their successful use in various systems, including yeast, *Drosophila*, rats and in human cells [24].

While aptamers consisting of natural RNA have mainly been used for intracellular applications, chemically stabilized forms of RNA, which are resistant to degradation by nucleases, have been used extensively to validate extracellular targets. 2′-fluoro-, 2′-amino- and 2′-O-methoxy-nucleotides are the most common modifications to increase the stability of RNA aptamers [25]. Alternatively, highly stable nucleic acid ligands can be obtained with L-ribose oligonucleotides, the so-called 'spiegelmers' [26,27]. Finally, a general advantage of aptamers as investigational tools in biological systems is their low toxicity and low immunogenicity [28].

Validation of extracellular targets

Vascular endothelial growth factor (VEGF) plays a significant role in angioneogenesis, tumor growth, and development [29–32]. Although the expression of VEGF has been observed in the kidney of rats and humans (as well as other tissues), little evidence exists for the role of renal VEGF in physiological or pathophysiological processes in the kidney, apart from the characterization of expression levels. Chemically stabilized nucleic acid ligands for $VEGF_{165}$ – the main isoform produced in glomerular cells – were isolated by *in vitro* selection [13] and used in *in vivo* rat models to gain further insights into possible functions of VEGF. After coupling to a 40kDa polyethyleneglycol (PEG) conjugate to prevent clearance from the blood, the aptamer antagonist was administered to healthy rats and rats with either mesangioproliferative nephritis, passive Heymann nephritis (PHN) or puromycin aminonucleoside nephrosis (PAN) [33]. The anti-VEGF aptamer had no effect in healthy rats following administration for 21 days, contradicting the previously presumed role of VEGF in the normal turnover rate of endothelial cells in the kidney. However, regeneration of glomerular endothelia in nephritic rats was significantly reduced by the PEG-conjugated aptamer compared with rats receiving either PEG alone or a PEG-conjugated sequence-scrambled control RNA, thus establishing that $VEGF_{165}$ plays an important role in cellular repair in glomerular disease.

In another study, a specific anti-platelet-derived growth factor (PDGF)-B aptamer was used in a rat model to establish that release of the PDGF-B chain is a central pathogenic event in the development of mesangioproliferative glomerulonephritis [34]. The same aptamers were used in a further experiment to investigate the participation of

PDGF-B and TGF-β signaling pathways in the mesangioproliferative nephritis model [35]. These examples demonstrate the potential of target-specific aptamers in understanding the functions of growth factors and other proteins in normal and diseased states.

Delivery of aptamers into cells

As aptamers are nucleic acids they can be delivered into cells by standard physical methods such as electroporation or lipofection. In addition, RNA aptamers can be transcribed from expression cassettes within the cell if, for example, long-term administration in cell culture is desired, or transgenic animals are under investigation. To enable effective intracellular expression of small RNA antagonists such as aptamers, the expression vector should: (1) produce stable and correctly folded RNA; (2) have a high transcription rate to enable a measurable functional effect of the aptamer; and (3) localize the aptamer to the same subcellular compartment as the target.

Various strategies have used cellular RNA polymerase (pol) II, RNA pol III or viral promoters for intracellular expression of aptamers. Good *et al.* investigated the ability of the strong human U6 pol III promoter to express small RNAs in the nucleus of human 293 cells [36]. Expression reached more than 4×10^5 copies of RNA per cell, and *in situ* hybridization of the transcripts clearly showed their nuclear localization. Localization of aptamer sequences in the cytoplasm was achieved when the constructs were transcribed from highly processive pol II promoters, such as the human cytomegalovirus (CMV) or the Rous sarcoma virus (RSV) promoter. Besides facilitating cytoplasmic expression of the aptamers, an added advantage is that inducible pol II promoter constructs are also available. Using an inducible heat shock promoter that is fully active within minutes, Shi *et al.* demonstrated the feasibility of regulating expression both temporally and spatially [37]. However, the efficiency of delivering nucleic acids into cells by electroporation and lipofection strongly depends on the cell type. However, this problem can be overcome by using poxviruses, which replicate exclusively in the cytoplasm of the host cell [38]. The vaccinia-based expression system under the control of a T7-RNA polymerase promoter mediated high-level cytoplasmic expression in leukocytes.

Validation of intracellular targets

Methods such as lipofection and the successful implementation of vector systems that enable high-level expression of aptamers in both the nucleus and cytoplasm of cells, have made intracellular targets accessible for functional analysis using high-affinity and -specificity aptamers. Such strategies have been used to inhibit several HIV-1 proteins essential for the life cycle of the virus, for example, Rev, which is an early HIV gene product required for viral replication. By interacting with the Rev binding site in the HIV viral genome, Rev regulates the export of viral transcripts from the nucleus into the cytoplasm, thus controlling the translation of viral structural proteins. Anti-Rev aptamers were recently shown to exhibit a specific and dose-dependent inhibition of HIV replication in cell culture [29,36,39]. Interestingly, this effect depended on the promoter used for intracellular aptamer (also known as intramer) expression: HIV replication was inhibited by 70% and 88% using pol III- and pol II-dependent expression vectors, respectively [39,40]. Similar studies have used aptamers against the HIV Tat protein and the RNaseH domain of the reverse transcriptase of HIV [23,41]: viral replication was efficiently inhibited by the nucleic acid ligands directed against both proteins.

Recently, an anti-idiotype approach was used to characterize the nuclear transport receptor (CRM1) recognition domain for the nuclear export signal (NES) of Rev [42]. An anti-Rev antibody able to block Rev-mediated export efficiently was used to select an RNA aptamer, which in turn was shown to bind specifically to CRM1. The strength of this interaction permitted the putative NES-recognition domain of CRM1 to be mapped to a central region of 108 amino acids. These results provide strong evidence that anti-idiotype RNA aptamers are valuable tools in both the identification and characterization of protein–protein interactions, and in the generation of inhibitors that specifically disrupt these interactions.

Blind *et al.* and Mayer *et al.* used aptamers expressed by the vaccinia virus-based T7-RNA polymerase expression system to gain new and decisive biological information about the intercellular adhesion molecule-1–leukocyte function-associated antigen-1 (ICAM-1–LFA-1) adhesion system [38,43]. Cytohesin-1 is a central activator of β2 (CD18) integrins on leukocytes and therefore plays a crucial role in the regulation of cellular adhesion [44]. When expressed in the cytoplasm of T cells, aptamers selected against either the intracellular domain of the human β2 integrin LFA-1 or the Sec7 sub-domain of its interacting partner cytohesin 1 (cyh1) specifically inhibited LFA-1-mediated T-cell adhesion to ICAM-1. Moreover, aptamers that inhibited the guanine nucleotide exchange activity of the Sec7 domain of cyh1 induced a cell-spreading deficient phenotype, thus establishing cyh1 as a modulator of T-cell cytoskeleton organization.

The effect of aptamers in a multicellular organism – the fruitfly *Drosophila melanogaster* – was demonstrated by Lis and colleagues [37]. The aptamer used was directed against

B52, a member of the *D. melanogaster* Ser-Arg-rich (SR) protein family comprising a class of nuclear proteins essential for pre-mRNA splicing and influencing splice site choice [45]. A multivalent pentameric form of the anti-B52 intramer was expressed under the control of a strong yet tightly controllable heat-shock promoter enabling dose-dependent investigation of intramer-mediated B52 counter-inhibition. Using the development of salivary glands and bristles in adult animals as morphological markers, Lis and colleagues demonstrated suppression of B52 overproduction by co-expression of the intramer. Moreover, *in situ* hybridization showed co-localization of B52 with the intramer, clearly indicating the interaction of both binding partners *in vivo*.

Aptamers as substitute ligands in HTS assays

Once an aptamer has been shown to antagonize the function of a target protein specifically, it can act as a substitute interaction partner in HTS assays to facilitate the competitive identification of small-molecule lead compounds. Connecting these two previously independent processes should result in significant streamlining of the drug discovery process. Aptamer-mediated bridging of target validation and HTS screening not only reduces the time required for proceeding from protein candidate to small molecule, but also provides the researcher with an unlimited number of substitute interaction partners for proteins with no known natural binding partner. Moreover, owing to the straightforward modification potential of nucleic acids, a broad range of screening strategies can be pursued, such as fluorescence polarization (FP), fluorescence resonance energy transfer (FRET) or bead-based assay systems. First examples have demonstrated that biologically active lead compounds can be identified through aptamer-based screening assays [46,47].

HTS-compatible molecular sensors

Nucleic acid ligands can be efficiently incorporated into HTS assays because they offer various starting points in the design of fluorescent read-out formats. The core binding domains of aptamers are usually between 20 and 40 nucleotides long. The straightforward structural characterization and the convenient incorporation of fluorescent dyes or affinity tags by standard oligonucleotide synthesis has been exploited to design ELISA-like formats [48], fluorescence quenching assays [49], fluorescence polarization assays [14] or ligand-dependent molecular beacons [50]. Figure 2 shows a schematic of an aptamer-based fluorescence polarization assay.

Another very sensitive detection assay can be designed with signaling molecules that combine the ligand-binding

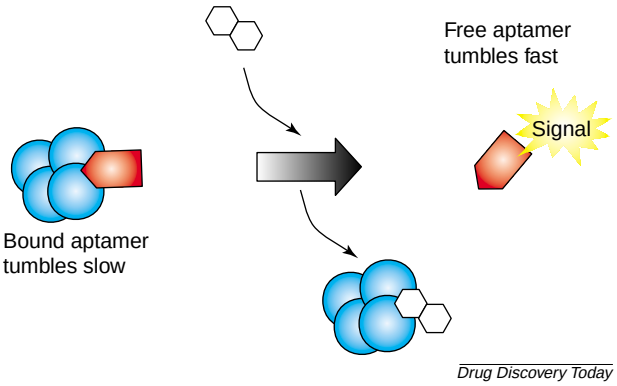

Figure 2. Schematic representation of an aptamer-based fluorescence polarization assay. Competition of the bound aptamer from its cognate protein target by a small-molecule competitor results in a change in fluorescence polarization.

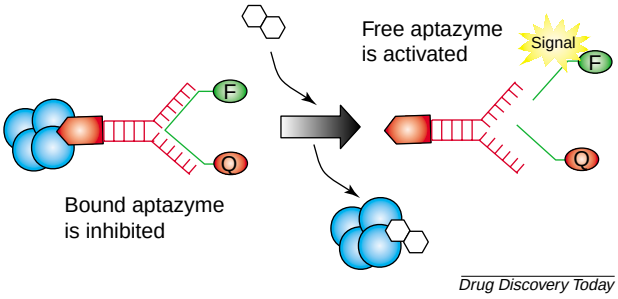

Figure 3. Schematic representation of an aptazyme-based screening assay. A protein-binding aptamer is fused to helix II of the hammerhead ribozyme to generate a protein-responsive ribozyme. Addition of the protein triggers a conformational switch within the aptameric domain, ultimately rendering the ribozyme inactive so that a fluorescence resonance energy transfer (FRET)-labeled substrate containing a fluorescent reporter (F) and a quencher (Q) remains uncleaved. Cleavage activity is restored in the presence of a small molecule that competes with the protein for binding to the aptamer. Depending on the design of the molecule, the catalytic activity of the ribozyme can also be activated upon protein binding.

capacities of aptamers with the catalytic properties of ribozymes (i.e. aptazymes). This combination of ribozyme sequences, whose catalytic activity (e.g. cleaving oligonucleotide substrates) is regulated by an incorporated aptamer sequence, was introduced by Breaker and colleagues [51,52]. The aptameric domain transduces structural changes to the ribozyme domain and activates or deactivates the catalytic activity depending on the design of the molecule (Fig. 3). The modulated activity can be detected by short oligonucleotide substrates carrying fluorescence reporter and quencher moieties that are subsequently separated by the cleavage reaction. Famulok and colleagues dem onstrated the compatibility of this cleavage reaction with HTS formats

Table 1. Small-molecule competitors used in the PDGF competition assay by Green *et al*.*

Competitor	K_{dC} [μM]	K_i [μM]
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
Amaranth	48 ± 16	26 ± 3.9
Sulfonazo III	74 ± 27	40 ± 19

*Abbreviations: K_{dc}, dissociation constant of competitor calculated from competition profiles; K_i, inhibition constant derived from [^{3}H]-thymidine incorporation assay; PDGF, platelet-derived growth factor.
Data taken from Ref. [46].

using 96-well plates [53]. The assay has several advantages, the first being its high sensitivity owing to the enzymatic turnover of the fluorescent substrate. In addition, it has a convenient, standardized read-out format because the general detection unit can be combined with any aptameric interaction domain.

Small-molecule inhibitors of PDGF BB

Janjic and colleagues designed a competition experiment based on DNA aptamers that specifically recognize the PDGF-B chain, and thus inhibit the binding of the growth factor to its receptor [54]. The experiment evaluated the ability of small organic compounds – which inhibit the binding of the PDGF-BB homodimer to its receptors – to displace the aptamer from its binding site [46]. The dissociation constant of the nucleic acid ligand was artificially lowered 10-fold to 0.65 nM by truncating the original aptamer to 27 nucleotides, thereby permitting the detection of small-molecule competitors with K_d values in the micromolar range. Twelve organic compounds were tested in radioactive filter binding studies for their ability to displace the aptamer from its target PDGF-BB. The small molecules were subsequently tested for their ability to inhibit PDGF-induced 3H-thymidine incorporation in rat A10 smooth muscle cells. The results showed that the K_{dC} values of the competition profiles and the K_i values in cell culture corresponded surprisingly well (Table 1). These data therefore indicate that biologically active aptamer probes are precise tools for predicting the biological activity of small-molecule inhibitors.

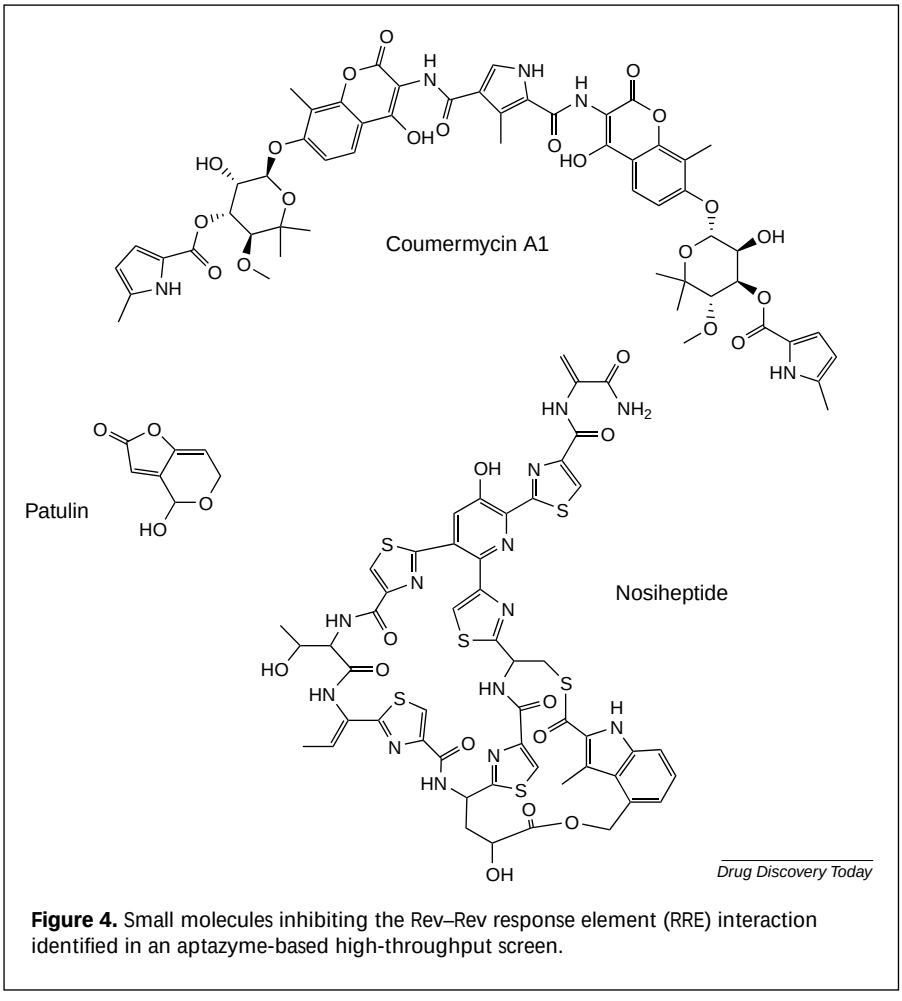

Figure 4. Small molecules inhibiting the Rev–Rev response element (RRE) interaction identified in an aptazyme-based high-throughput screen.

Small-molecule inhibitors of HIV replication

Recently, the concept of using nucleic acid inhibitors as substitute ligands for identifying novel small-molecule inhibitors was comprehensively demonstrated by Famulok and colleagues [47]. An inhibitory aptamer of the HIV-1 Rev protein [36] and the minimal Rev binding element (RBE) [55] were used to construct a set of two Rev-responsive aptazymes. These aptazymes either activated or inhibited cleavage of a 13-nucleotide FRET-labeled oligonucleotide substrate with a fluorescent reporter (carboxyfluorescein or FAM) at the 5′ end and a quencher (carboxytetramethylrhodamine or TAMRA) at the 3′ end. A small library of structurally diverse antibiotics was screened in aptazyme-based ligand displacement assays for novel inhibitors of HIV replication.

Three compounds were identified that were able to reverse the Rev-dependent ribozyme activity (Fig. 4). The K_i values of these hits determined by surface plasmon resonance analysis ranged from 5.4 μM to 18.5 μM. It was shown that the compounds disrupted the protein–aptazyme interaction by binding to the Rev protein, none of them mediating its effect by interaction with the aptazyme reporter. Coumermycin A1, the most promising candidate from the *in vitro* binding studies, was further analyzed in an HIV replication assay. In cell culture, p24 concentrations (an indicator of HIV replication) were reduced to undetectable levels at an antibiotic concentration of 7.5 μM (Fig. 5), showing that an inhibitory aptamer with antiviral activity in cell culture [36] is a useful tool for identifying functionally equivalent small-molecule lead compounds.

Concluding remarks

The unique features of aptamers directly bridge the gap between target validation and the subsequent process of HTS. These nucleic acid bio-tools are valuable substitute compounds for identifying relevant sites on potential target proteins. The ease of automatically selecting aptamers, their straightforward use in biological read-out systems and their subsequent applications in HTS to discover competing, functionally equivalent small-molecule lead compounds perfectly match the demands made on technologies accelerating the drug discovery process. Without doubt, aptamer-based technologies provide a convenient and time-saving approach to converting functional proteomic data into concrete lead compounds.

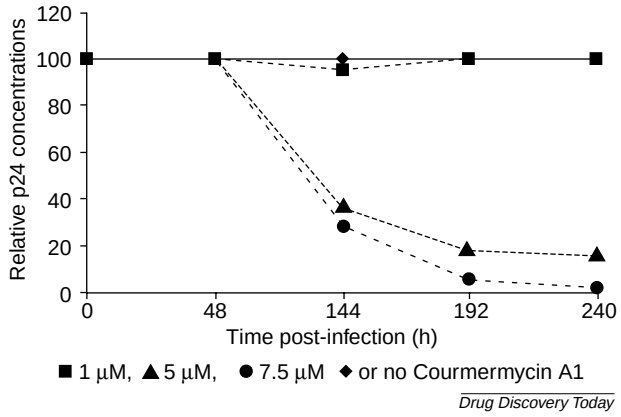

Figure 5. Inhibition of HIV-1 replication by Coumermycin A1. H9 cells were infected with HIV-1_{IIIb} and grown in the presence of either 1 μM (filled squares), 5 μM (filled triangle) or 7.5 μM (filled circles) coumermycin A1, or without coumermycin A1 (filled diamonds). Culture supernatants were collected over the course of ten days and assayed for p24 concentration. p24 concentrations are shown relative to infected cells treated with similar volumes of DMSO.

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