Intramers and Aptamers: Applications in Protein-Function Analyses and Potential for Drug Screening

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1. Introduction

During the past decade, the complete genomes of more than 140 different organisms have been sequenced and made available in databases.[1–4] These databases provide extremely useful collections of organised, validated data, which are indispensable for genomics and proteomics research and the drug-discovery process. Differential analyses of pathogenic and healthy states of organisms and/or isolated cells provide a picture of genes and gene products that are related to, or actually responsible for, defined diseases. The challenge today is to understand in detail the function and interplay of the numerous proteins in different organisms, tissues, cell types and conglomerate protein complexes.

Among the most effective ways to study the function of a given protein in the context of the living cell or organism is the application of a small-molecule drug that exhibits high specificity, affinity and inhibitory activity for the protein under investigation. However, because such inhibitors are available only for a minority of the estimated total number of proteins of higher vertebrate organisms,[5,6] protein function is most commonly studied by loss-of-function phenotypic analysis.

2. Loss-of-Function Phenotypic Analyses at the mRNA Level

Most traditional approaches for this purpose usually rely on observation of phenotypic alterations of a cell or organism as a consequence of alteration of its genetic information. In general, this is achieved either by transgenic knockout technologies[7] or by dominant negative expression of a protein or a mutant derivative. However, genome manipulation is a time-consuming and expensive approach, requiring invasive intervention.

A less laborious alternative is to gain functional information by targeted mRNA destruction of the gene of interest (Figure 1). This can be achieved, for example, by antisense oligodeoxynucleotides (ODNs)[8]—ssDNA—or chemically modified oligonucleotides[9] with nucleotide sequences that are complementary to the mRNA to allow sequence-specific hybridisation. Protein production is then blocked either by inhibition of ribosome scanning of the mRNA or by activation of endogenous RNase H, which recognises these heteroduplexes and hydrolyses the mRNA part (Figure 1 b). Problems associated with the antisense approach are that many ODNs often exhibit an intolerable degree of toxicity and that their target sequences on

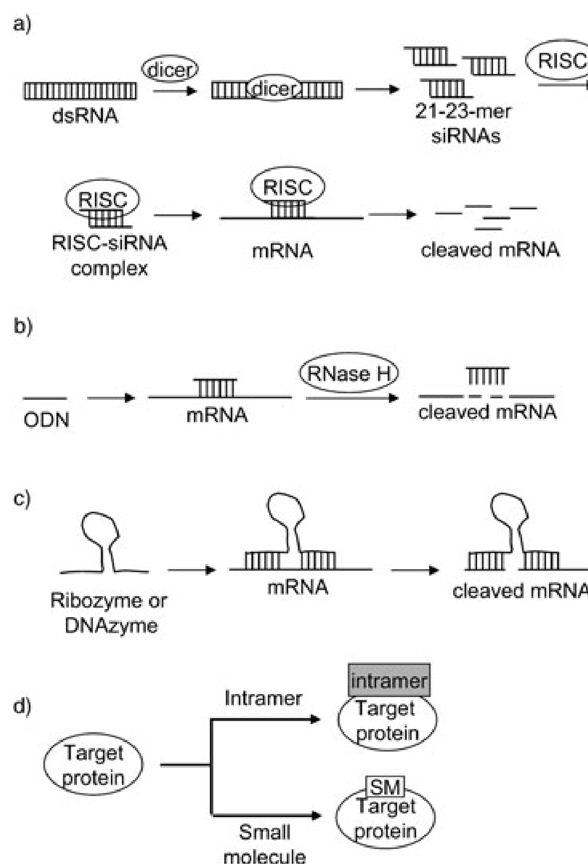

Figure 1. Mechanisms of phenotypic knock-down at the level of mRNA stability or by direct inhibition of the protein. a) The siRNA processing machinery. A longer double-stranded RNA molecule is processed by dicer into short interfering RNAs (siRNAs) of 21–23 nucleotides in length. siRNAs are bound by proteins of the RNA-induced silencing complex (RISC). The RISC–siRNA complex directs the antisense strand of the bound siRNA to a region on the target mRNA that is exactly complementary, inducing destruction of the mRNA target and preventing the target protein from being made. b) Major mechanism of mRNA degradation by antisense oligodeoxynucleotides (ODNs). The ODN hybridises to its target sequence on the mRNA, which is cleaved by endogenous RNase H. c) Schematic for mRNA cleavage by intracellular ribozymes or deoxyribozymes (DNAzymes). d) Direct inhibition of the target protein by intramers or by small molecules.

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mRNAs may be inaccessible due to bound proteins or because the mRNA is engaged in higher-order structures[10]

Other options employed for similar purposes are intracellular ribozymes[11,12] or DNAzymes.[13–15] Unlike ODNs, ribozymes have the advantage of cleaving the target mRNA with multiple turnover, while their mechanism of recognition of their target mRNA sequence also operates through simple Watson–Crick pairing (Figure 1 c). As enzymes that cleave phosphodiester bonds they are independent of the host-cell's endogenous RNase activity. Several examples have shown that intracellularly expressed ribozymes can efficiently down-regulate the expression of proteins; they have been extensively reviewed elsewhere.[11,12,16–20] With regard to cleavage-site selection, ribozymes and DNAzymes face similar problems to ODNs, and some impressive endeavours have been successfully undertaken to overcome these obstacles.[21–23]

In the past few years, another extremely versatile method for silencing genes on the mRNA level has become available, in the form of short interfering RNAs (siRNAs). siRNAs are RNA double strands of 21–22 nucleotides in length that can down-regulate the expression of eukaryotic genes with complementary sequences by utilising the RNA-induced silencing complex (RISC) protein components of the RNA interference (RNAi) machinery (Figure 1 a).[24] Short interfering RNAs have emerged as a powerful laboratory tool for knocking down gene expression in various cells and organisms, because their design is simple and because they can be easily obtained by standard RNA synthesis, thus allowing straightforward analysis of biological functions of specific genes. Their application potential is wide and, like intracellular ribozymes, siRNAs can be endogenously expressed in a variety of cells, as summarised in several review articles.[20,25–31]

3. Analysis of Protein Function with Inhibitors

While all these approaches have proved invaluable as tools for functional genomics, they share certain disadvantages associated with the alteration of the amount of an expressed protein in the context of its natural functional network in a cell, tissue or organism. Alteration of the genetic information of an organism often has secondary effects on the expression pattern of other genes in a somewhat unpredictable fashion. Also, siRNAs, useful and versatile as they are, sometimes only give partial knock-down of their target protein or can result in the undesired induction of interferon response,[32] which may hamper an unbiased analysis of gene function.

Specific modulation of gene function at the protein level is therefore still highly desirable. The post-genomics era and the need to develop novel pharmaceuticals have created a growing demand for specific ligands and inhibitors that will act directly on the protein or a defined protein subdomain without altering the genetic or mRNA status of an organism (Figure 1 d).[33–38] Direct inhibition of a protein allows immediate insight into questions such as drugability, or the functional role of sub-domains or post-translational modifications. The analysis of gene function on the protein level requires direct recognition and inhibition of protein targets by inhibitory molecules that need to fulfil certain criteria: they should be routinely obtainable and applicable independent of the target, and act at low concentrations, with high specificity and in an intracellular context. A class of molecules that fulfils these requirements is nucleic acid ligands, or aptamers.

Aptamers are short, single-stranded oligonucleotides that fold into distinct three-dimensional structures capable of binding their targets with high affinity and specificity, basically mediated by complementary shape interactions.[39–44] They can be isolated from vast combinatorial sequence libraries by SELEX (systematic evolution of ligands by exponential enrichment), an in vitro selection process.[45,46] The SELEX method has been applied to many different targets ranging from small organic molecules[47] to large proteins[43] and even viruses[48,49] or parasites.[50] Moreover, in most cases aptamers not only bind their cognate protein but also efficiently inhibit its function. Thus, aptamers represent an interesting compound class that can be easily obtained and used for assessing the function of a defined protein target. In fact, owing to the increasing demand for protein inhibitors in the post-genome era, selection routines compatible with automation have been established that allow highly parallel aptamer selections to several targets at once to be carried out within a few days.[51–53]

A large number of aptamers have been selected for preferential targeting of extracellular proteins or protein epitopes. This is not surprising, because this way they have direct access to their targets without having to pass through plasma or nuclear membranes. However, for them to be broadly applicable as inhibitory tools for functional genomics research, it is necessary to develop methods that allow for targeting of proteins that reside inside cells with inhibitory aptamers (hereafter designated as intramers). Being nucleic acids, intramers are intrinsically adapted to the reductive environment inside a cell—unlike, for example, intracellular antibodies (intrabodies), which require further engineering to tolerate the reductive conditions of the cytoplasm. The cellular delivery of aptamers can be accomplished either by direct transfection or through defined expression systems encoding for the aptamer sequence under the control of a highly active promoter. Below we summarise recent progress made in intramer technology.

4. Intramers Targeting Nucleic Acid Binding Proteins

The first examples of the functional characterisation of intramers inside cells included prokaryotic or nuclear targets.[54–57] In most cases, aptamers that targeted natural nucleic acid binding proteins were selected in vitro and intramer-expressing systems were engineered afterwards. Expressed intramers often acted as decoys to natural RNA-binding proteins or represented variants of natural transcripts. Their intracellular expression was intended to dissect functional aspects of nucleic acid binding proteins or of structural elements in natural transcripts.[20,35,44,58]

A good example is an aptamer that binds the *Drosophila melanogaster* B52 protein, a splicing factor essential for pre-mRNA splicing in fruit flies. Fly mutants expressing B52 above

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or below certain levels have developmental defects or lethal phenotypes. In transgenic flies that expressed a pentameric version of the aptamer, developmental defects caused by overexpression of B52 were rescued by the intramer, while intramer expression in a wild-type B52 strain was lethal. Other proteins targeted with intramers included the *E. coli* special elongation factor SelB, required for incorporation of the amino acid selenocysteine into selenoproteins,[54,59] yeast RNA polymerase II[55] and several viral proteins. Among them, aptamers that target the HIV-1 Rev protein showed Rev-binding affinity similar to that of the wild-type Rev-binding element (RBE). In cell cultures, these aptamers supported the Rev-dependent pre-mRNA transport from the nucleus to the cytoplasm.[57]

More recently, Chaloin et al. have reported the expression of an HIV-1 reverse transcriptase (RT) binding RNA aptamer pseudoknot inside human 293T cells.[60] Specifically, 293T cells were transiently transfected with a chimeric RNA expression system consisting of the human $tRNA^{Met}$ and the anti HIV-1 RT RNA aptamer module. Expression of the anti-RT intramer resulted in inhibition of HIV particle release by $>75\%$ when the cells were co-transfected with proviral HIV-1 DNA. Subsequently, HIV-1 particles produced by 293T cells in the presence of the anti-RT intramer construct exhibited reduced infectivity in human T-lymphoid cells. Again, virus production was reduced by 75% relative to control experiments. Complete inhibition of viral replication was achieved in stably transfected T-lymphoid cells after low-dose HIV infection over a period of 35 days.[60]

Similarly, Kim and Jeong also aimed at inhibition of HIV-1 replication by RNA intramers by targeting the nucleocapsid (NC) protein. In vitro, an anti-NC aptamer interfered with NC binding to the stable transactivation response (TAR) hairpin and psi RNA stem-loops of HIV-1 RNA. In vivo, the aptamer abolished packaging of viral genomic RNA.[61] Inhibition of viral replication by intramers was also achieved when proteins from viruses other than HIV-1 were targeted. The Nishikawa group targeted the non-structural protein NS3 of hepatitis C virus (HCV), a protein with both helicase and protease activity. They demonstrated that protease activity was inhibited through intracellular expression of the aptamer.[62]

Further studies underline the feasibility of the intramer concept for targeting proteins in artificial systems. Burke and colleagues targeted HIV-1 RT in *E. coli* mutants that were genetically engineered to depend on reverse transcriptase for growth at 37 °C.[63] It was shown that growth complementation by the expressed exogenous HIV-1 RT can be blocked by anti-RT intramer expression.

Cassiday and Maher selected an RNA aptamer targeting the transcription factor NF-κB. This aptamer was shown to recognise NF-κB inside cells and to inhibit its binding to its cognate DNA sequence, presumably by acting as a mimic of the dsDNA motif.[64,65] To further optimise the formation of the NF-κB-RNA complex in the eukaryotic nucleus, a yeast three-hybrid system was used to re-select the RNA aptamer for improved NF-κB interaction[66] (Figure 2). By use either of a degenerate RNA library or of sequences from early selection cycles, aptamer variants with substantially improved binding affinity in yeast cells were obtained. Furthermore, the improved aptamer variant inhibited the transcriptional activity of NF-κB[66] in vivo. These results underline the power of the combination of in vitro and in vivo genetic selections for the optimisation of aptamer properties and their adaptation to distinct conditions.

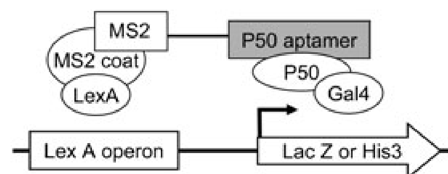

Figure 2. The yeast three-hybrid system used for the selection of anti-p50 aptamers with increased activity in relation to the in vitro selected parent aptamer. Transcription of lacZ or HIS3 reporter genes depends on the interaction between the LexA/MS2 coat protein fusion, a hybrid RNA composed of the MS2 recognition sequence and the α-p50 aptamer sequence, and a GAL4/p50 hybrid protein.

Within the past several years, substantial progress has been made in the field of intramer research. Besides reports of novel intramers and their effective inhibition of their cognate targets inside cells, a few studies have compared in vitro with in vivo results and the adaption of aptamers for in vivo functionality. For example, a study by Lee and McClain provided evidence that results obtained with functional RNA sequences in vitro have to be carefully validated in vivo and cannot necessarily be adapted to in vivo conditions.[67] Lee and McClain used a $tRNA^{Gln}$ variant selected in vitro and exhibiting an affinity for glutaminyl-tRNA synthetase 26 times higher than that of the wild-type $tRNA^{Gln}$. These variants can be efficiently aminoacylated in vitro by glutaminyl-tRNA synthetase. Nevertheless, they failed to support the growth of a *E. coli* $tRNA^{Gln}$ knockout strain in vivo because, unlike wild-type $tRNA^{Gln}$, they were rapidly degraded by cellular RNases. This result underlines the importance of gaining both in vitro and in vivo data on macromolecular function before conclusions on biological relevance can be drawn.

5. Elucidating Novel Biological Activities of Proteins with Intramers

Our research led us to the development of highly specific aptamers targeting cytoplasmic regulatory proteins and protein domains implicated in the leukocyte function associated antigen-1 (LFA-1) mediated inside-out signalling cascade. The activation of LFA-1 by T-cell receptor stimulation or stimulation with phorbol esters results in T-cell adhesion to ICAM-1 presented on the surface of endothelial cells. Cytohesin 1, a cytoplasmic signalling molecule, participates in the mechanism of LFA-1 activation, presumably by direct interaction with the cytoplasmic tail of the β2-chain (CD18) of the LFA-1 integrin.[68] Cytohesin 1 belongs to a family of highly homologous guanine nucleotide exchange factors (GEFs) that act on ADP-ribosylation factors (ARFs). The small ARF-GEFs are known to be involved in integrin signalling, actin cytoskeleton remodelling and vesicle transport. Today, four highly homologous members of the cytohesin family are known: cytohesin 1, ARNO/cytohesin 2, cytohesin 3 and cytohesin 4. They comprise an N-termi-

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nal coiled-coil domain, followed by a Sec7 and pleckstrin homology domain, and a polybasic C-domain. To dissect their individual functions, we isolated RNA aptamers that specifically interact with cytohesin proteins and/or their individual domains and thus allow dissection of their functions in living cells. In an initial study we isolated and characterised the cytohesin 1 binding aptamers M69 (Figure 3a). M69 specifically recognised the Sec7 domain of cytohesin 1, which is responsible for the GEF activity and is thought to interact directly with the β2-cytoplasmic tail of LFA-1. Sec7 domains are widespread within the small and large families of ARF-GEFs. M69 distinguished between the Sec7 domains of the large and small GEF family members, by binding only to those of the small GEFs ARNO and cytohesin 1. However, it did not discriminate between the Sec7 domains of these two cytohesins.[69]

An expression system based on transgenic vaccinia viruses[70] was used for cytoplasmic expression of M69 in Jurkat cells.[69] Intrameric expression of M69 caused inhibition of LFA-1-mediated adhesion. Furthermore, the intramer M69 inhibited reorganisation of the cytoskeleton and cell spreading. Dominant negative expression of a GEF-deficient cytohesin 1 (E157 K) point mutant gave similar results, confirming an important role for the GEF activity of cytohesin 1 in T-cell spreading.

Do the highly homologous cytohesins 1 and 2 exhibit different functions in T-cells in which they are both expressed? As mentioned above, M69 did not discriminate between the highly homologous cytohesin family members. To address this question, we performed an in vitro selection with ARNO/cytohesin 2 as a target and cytohesin 1 in a counter-selection, to isolate discriminatory aptamers. We obtained an RNA aptamer, dubbed K61 (Figure 3b), that bound ARNO/cytohesin 2 with a K_D of 115 nM, whereas cytohesin 1 was bound with an affinity at least 35 times weaker. This aptamer did not inhibit the GEF-activity of ARNO in vitro, presumably because it recognises the coiled-coil–Sec7 interface of ARNO/cytohesin 2, exhibiting only weak affinity to the Sec7 domain alone.[71]

GTPases of the Rho and ARF families are thought to regulate membrane traffic and cytoskeletal remodelling.[72] Furthermore, the Rho family of small GTPases is involved in the stimulation of serum response factor transcriptional activation, induced by serum growth factors.[73] We thus hypothesised that cytohesins may also play a role in transcriptional activation through the serum-response element (SRE) and investigated whether K61 affects the transcription of a luciferase reporter gene under the control of the SRE promoter in serum-stimulated HeLa cells (Figure 3c). We found that K61 down-regulates SRE-controlled luciferase expression to basal levels in a concentration-dependent manner. The inhibitory activity of K61 was fully reversed by over-expressed wild-type ARNO, confirming that the effect was aptamer-specific.[71] In accordance with this novel activity

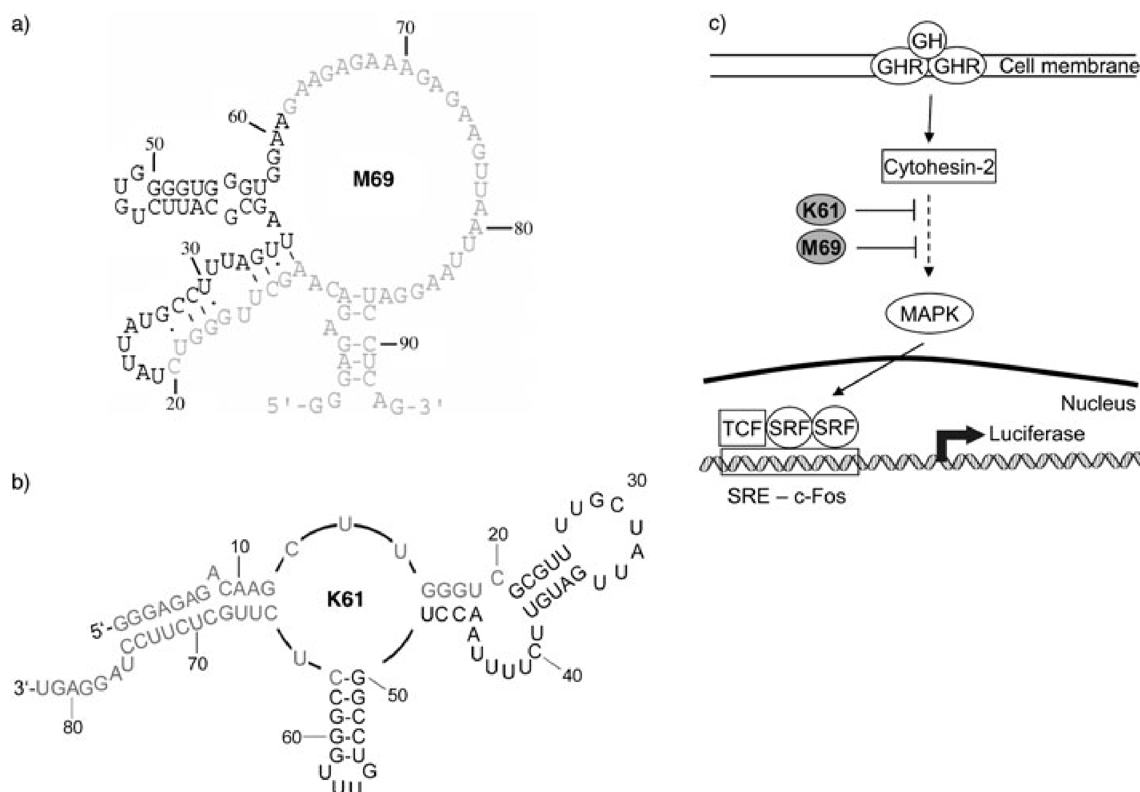

Figure 3. Using intramers to confer novel biological function on cytoplasmic signalling molecule cytohesin 2 in HeLa cells. a) Proposed secondary structure of aptamer M69, which is specific for the Sec7-domains of the small GEF family members cytohesin 1 and cytohesin 2. b) Proposed secondary structure of the cytohesin 2-selective aptamer K61. c) Model for the role of ARNO/cytohesin 2 as an effector of serum-mediated transcriptional activation through MAPK signalling in HeLa cells. The domain specificity of K61 and M69 suggest participation of both the N-terminal and the Sec7 domains of ARNO/cytohesin 2 in this activity (S: growth factors in serum that act on MAPK-activating receptors; SR: MAPK-activating receptors; CC: coiled-coil domain of cytohesin 2).

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of cytohesin 2, we found that both K61 and the non-discriminatory aptamer M69 produced a specific down-regulation of MAPK activation, as monitored by the phosphorylation status of Elk. An siRNA-targeting cytohesin 2 also resulted in the down-regulation of MAPK activation, but interestingly, an siRNA that down-regulated cytohesin 1 expression did not. These results suggested that transcriptional regulation of the SRE in HeLa cells could be assigned to ARNO rather than to cytohesin 1. This study further demonstrated that intramers can be used to provide insight into novel biological activities of target proteins and to assign specific biological functions to individual members or defined domains of a protein family. Intramers represent an alternative and complementary approach to siRNAs to elucidate the function of a protein within its natural context.

6. Aptamers as Probes for Screening Approaches

The results described above show that aptamer selections provide a versatile source for obtaining powerful inhibitors of protein function within short timescales. Once an inhibitory aptamer is available, it is fairly straightforward to transform it into an intramer for in vivo validation of protein function. In fact, the anti-ARNO aptamer K61 was directly transfected into cultured cells by lipofection without the need to stabilise it against nuclease degradation or to direct it into a particular cellular compartment.[71] The question is how general this approach will be with respect to cell types, targets, or the aptamer sequence itself. The analysis of protein function in multicellular organisms or tissues will necessitate the generation of aptamer-expressing transgenic animals or the development of gene therapeutic approaches, at least in the case of vertebrate studies. For such purposes, drug-quality small-molecule inhibitors would still seem advantageous compared to any nucleic acid- or biopolymer-based inhibitor.

Therefore, one intriguing idea is to convert an aptamer/protein complex with verified intracellular functionality directly into lead compounds by developing assays to screen small-molecule libraries that displace the aptamer from its target and adopt its inhibitory activity. Indeed, aptamers would seem perfectly suited for functioning directly as competitive probes in high-throughput screening (HTS) assays. This would allow direct translation of information stored within an aptamer into a small molecule, which would itself be likely to be an inhibitor (Figure 4a).

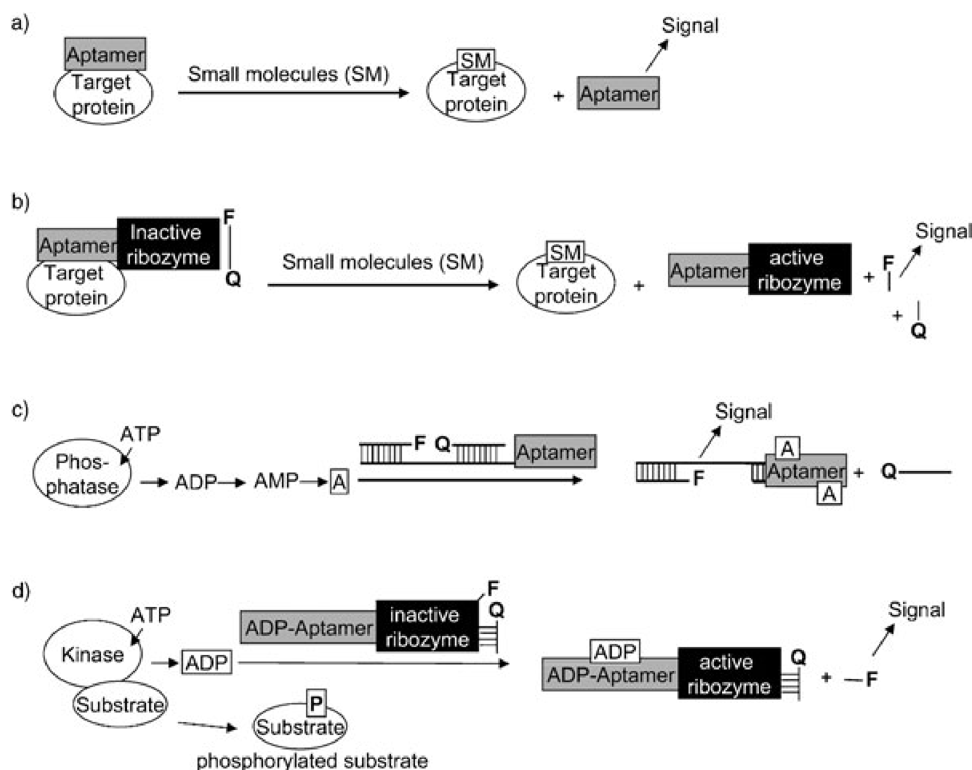

Figure 4. *Aptamers for inhibitor screening. a) Schematic for how a functional aptamer/target complex might be used to develop screening assays to allow identification of small molecules that displace the aptamer from the target, resulting in a signal. b) Reporter ribozymes for high-throughput screening.[74,75] If a small molecule can compete with the protein/RNA interaction, the ribozyme becomes active and cleaves a substrate, labelled with a fluorescence tag (F) and a quencher (Q). In the uncleaved state, the substrate has a low fluorescence quantum yield, due to fluorescence resonance energy transfer (FRET). In the cleaved state, the two product oligonucleotides rapidly dissociate from the ribozyme, resulting in a fluorescence signal. These reporter ribozymes were used to identify a novel small-molecule inhibitor for the HIV-1 Rev protein. c) Signalling aptamers for monitoring of enzyme activity.[90] The DNA aptamer reports the presence of two molecules of adenosine (A), generated by dephosphorylation of ATP, ADP or AMP with alkaline phosphatase. In the presence of A, the aptamer folds such that the quencher-labelled oligonucleotide can no longer bind to the signalling aptamer construct. In the absence of A, the signalling aptamer has a low fluorescence quantum yield, due to FRET resulting from the close proximity of the hybridised fluorescence- (F) and quencher-labelled (Q) DNAs. These probes can report known inhibitors of alkaline phosphatase. d) ADP-specific RiboReporters.[92] ADP is generated upon substrate phosphorylation by a kinase from an ATP cofactor, and specifically bound by the ADP aptamer module, making the ribozyme module active towards cleaving off the F-labelled product oligonucleotide. In the inactive form in the absence of ADP, the RiboReporter has a low fluorescence quantum yield because of the close proximity of the F-label to a hybridised Q-labelled DNA. After cleavage, the F-labelled product dissociates from the ribozyme, emitting a fluorescence signal.*

As a first step towards this direction, we have developed what we called "reporter ribozymes": chimeric RNA molecules that consist of an aptamer domain attached to a ribozyme.[74,75] Reporter ribozymes are potentially compatible with the parallel screening of large compound libraries since they report the displacement of a protein-bound aptamer by a small molecule

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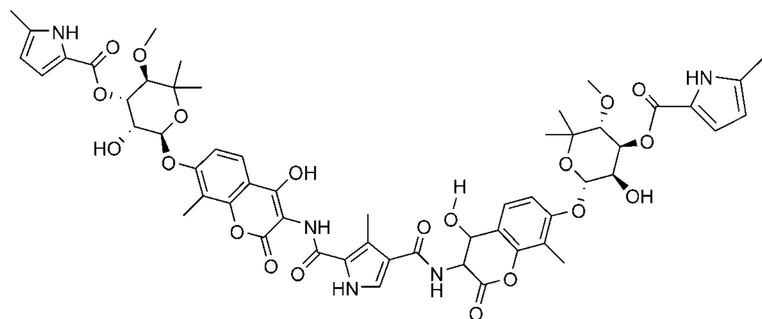
through a fluorescence signal (Figure 4b). The detection principle relies on an intrinsic property of aptamers and many natural protein-binding RNAs: adaptive binding. Ever since the first NMR structures of aptamer/small-molecule complexes were elucidated it has been clear that the complexation of a ligand by an aptamer occurs almost exclusively through adaptive recognition. That is, for example, aptamers often comprise unpaired loop or bulge regions, which are disordered in the free nucleic acid and acquire a defined conformation through adaptive folding around the ligand.[76] The target of the reporter ribozymes was the Rev protein of HIV-1. Binding of Rev to its cognate natural RNA element, the Rev-responsive element (RRE) in the reporter ribozyme rendered the ribozyme module inactive. Only when the aptamer was competed by a Rev-binding small molecule did the ribozyme module undergo conformational changes enabling it to cleave a substrate oligonucleotide possessing a fluorophore at one end and a quencher at the other. Fluorescence was detected only when the ribozyme was active (Figure 4b). Conversely, an alternative ribozyme construct containing a Rev-binding aptamer[77] module showed exactly the opposite behaviour, being switched on in the presence of Rev and switched off after Rev was subjected to competition by a small molecule.

The Rev-responsive reporter ribozyme was used to screen a 96-member sample library of antibiotics for molecules that could disrupt the interaction between Rev and its cognate RNA. The screen identified three compounds as hits; one—the gyrase inhibitor coumermycin A1 [*N*,*N*′-bis(7-((6-deoxy-5-*C*-methyl-4-*O*-methyl-3-*O*-((5-methyl-1*H*-pyrrol-2-yl)carbonyl)-α-L-lyxo-hexopyranosyl)oxy)-4-hydroxy-8-methyl-2-oxo-2*H*-1-benzopyran-3-yl)-3-methyl-1*H*-pyrrole-2,4-dicarboxamide]—exhibited fairly specific binding with a K_D of 7.5 μM. Moreover, cell culture experiments revealed that the coumermycin A1 inhibits the HIV-1 virus replication in a concentration-dependent fashion; this indicates that the small molecule possesses the same characteristics as the aptamer from which it was derived. This study established that it is possible to identify novel small molecule inhibitors for a given protein by using interference with RNA/protein interactions as a basis for screening.



In a similar study,[75] we fused an HIV-1 reverse transcriptase (RT) binding aptamer[78] to the hammerhead ribozyme. The presence of RT induces the formation of a different structure of the aptameric portion (i.e., a pseudoknot structure[79]). As in the study described above,[74] the binding of RT to the aptamer prevents the ribozyme from cleaving the small oligonucleotide substrate RNA, labelled with the fluorescent dye and the quencher. In the absence of RT the reporter ribozyme remains active, and substrate cleavage can be followed by an increase in fluorescence. This is highly specific for HIV-1 RT; the homologue RT of HIV-2 is not detected. The reporter ribozyme thus serves as a specific biosensor signalling the presence of HIV-1 RT. In this sense, reporter ribozymes supplement currently used antibody-based techniques, like ELISA, while being considerably more straightforward due to real-time readout in solution and other advantages discussed below. The assay is reversible: when the protein is displaced from the reporter ribozyme through interaction with another molecule, such as the primer/template complex that is mimicked by the aptamer, the reporter ribozyme can again cleave the substrate, resulting in a fluorescence signal. In other words, these systems can act as *domain-specific* sensors for screening purposes. As mentioned above, the reporter ribozyme binds HIV-1 RT at the same site at which the protein recognises the primer/template complex. Remarkably, RT-binding molecules that are specific for sites other than the primer/template binding site are ignored by the reporter ribozyme. This enables the configuration of an assay into a high-throughput screening mode that might allow a highly focused search for inhibitors that target a distinct epitope or domain of a protein. Most of the HIV-1 RT inhibitors known today are nucleotide RT inhibitors (NRTIs) such as azidothymidine and non-nucleotide RT inhibitors (NNRTIs) such as nevirapine, which target other domains of the polymerase. As a perspective, our approach would now allow the search for completely novel classes of HIV-1 RT inhibitors that target the primer/template binding site. This potential was tested by addition of the free aptamer as a specific competitor for the reporter ribozyme. The aptamer was able to displace the protein from the reporter ribozyme, switching it on again. Reporter ribozymes thus have three distinct advantages over many other assays or sensors: detection occurs in real time, none of the actual reaction partners need to be labelled, and the format is highly modular and can be configured for any kind of protein for which aptamers can be selected.

Further modularity of reporter ribozymes was achieved in another format, based on hairpin ribozyme variants that can be induced or repressed by external effector oligonucleotides. The key step here was to introduce a binding domain specific for a certain RNA sequence into the hairpin ribozyme. When the domain is bound by the cognate RNA, the reporter ribozyme undergoes conformational changes enabling it to cleave the fluorophore and quencher-labelled substrate oligonucleotide. Small sequence changes in the RNA-binding domain allowed targeted switching of ribozyme activity: the same effector oligonucleotide then serves either as an inducer or as a repressor. We applied this format to a hairpin variant fused to the complementary version of the trp leader mRNA,[80] the RNA sequence tightly bound by L-tryptophan-activated trp-RNA-binding attenuation protein (TRAP) from *Bacillus subti-*

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lis. TRAP only binds to trp leader mRNA in the presence of L-tryptophan.[81] Ribozyme activity can be altered by annealing with trp leader mRNA, and then specifically restored by its TRAP/tryptophan-mediated sequestration. These reporter ribozymes thus sense the activity status of a protein as a function of its metabolite molecule and could potentially be applied for screening of TRAP-binding small molecules. Using the same format, we designed nine ribozyme variants that were activated by different microRNAs (miRNAs).[82] Each of them detected its cognate miRNA reliably and sensitively in a mix of other miRNA sequences. These reporter ribozymes join other ribozymes that report nucleic acids.[83–86] They are entirely RNA-based and thus could be expressed endogenously, requiring only the addition of the short substrate oligonucleotide to report the presence of a certain miRNA in an in vivo context.

Green et al. screened a panel of naphthalenesulfonic acid anions for their ability to displace a ^{32}P-radiolabelled DNA aptamer from the platelet-derived growth factor (PDGF) B-chain.[87] The *anti*-PDGF-aptamer inhibits binding of PDGF to cells that express PDGF receptors.[88] Twelve organic anion derivatives, known to exhibit similar activity to that of the aptamer,[89] were analysed for their ability to disrupt the aptamer–PDGF complex. By using the small molecules in functional assays, it was shown that the binding affinities of all ligands tested (small molecules and aptamers) strongly correlated with their inhibitory potential.

Another elegant approach was developed by Nutiu et al., who also used aptamers as sensors for small-molecule metabolites, thereby allowing the monitoring of enzymatic reactions.[90] They used a DNA aptamer[91] with a higher affinity for adenosine than for 5′-adenosine monophosphate (AMP) as fluorescence reporter to quantify the yields of the ALP-catalysed (ALP = alkaline phosphatase) cleavage of AMP to adenosine (Figure 4 c). Furthermore, the aptamer reporters can be used for sensitive detection of ALP. The authors demonstrated that the applied aptamer reporters are useful as screening probes for the indirect identification of known small-molecule inhibitors of ALP.

Srinivasan et al. utilised an anti-ADP aptamer that discriminates between adenosine diphosphate (ADP) and adenosine triphosphate (ATP) to construct a ribozyme-based allosteric sensor, which they call “RiboReporter”, for monitoring kinase activities.[92] As in the studies by Hartig et al.,[74,75] detection relies on fluorescence and quencher-labelled oligonucleotides and their cleavage by the ribozyme. They also re-identified previously known kinase inhibitors in a proof-of-concept screening approach for kinase inhibitors (Figure 4 d).

Taken together, these results illustrate the potential of aptamers and aptamer-based sensor systems for the identification of small molecule inhibitors.

Summary

Aptamers are easily handled chemicals that can be isolated for various proteins by in vitro selection and selectively bind a large variety of different targets, from proteins or individual domains of homologous proteins to small molecules, viruses, cells and parasites. They can be used both as functional inhibitors to characterise proteins either inside or outside a cell and as tools to develop inhibitors for protein interference. Furthermore, it was recently discovered that nature harnesses allosteric binding of small-molecule regulators to aptamers contained in the untranslated regions in messenger RNAs of many bacteria, so-called riboswitches, to regulate gene expression.[93]

Aptamers offer a valuable complement to loss-of-function phenotypic knockdown approaches and the assignment of novel activities to members of highly homologous protein families. Moreover, besides their conventional uses as diagnostic reagents, affinity matrices or therapeutics, aptamers offer an exciting novel interface between target validation and drug screening, as a biologically active aptamer can be used to identify functionally equivalent small molecules directly in competitive high-throughput screening assays. We consider aptamers are a highly promising alternative to other tools for the loss-of-function phenotypic analysis of proteins for validation of the biological activity of new proteins inside cells, and for the development of novel chemical entities for the rapid characterisation of proteins in the context of whole cells or organisms.

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Keywords: aptamers · intramers · loss-of-function phenotypic analysis · proteomics · ribozymes · siRNA

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