Targeting RNA: new opportunities to address drugless targets

Guido J.R. Zaman, Paul J.A. Michiels and Constant A.A. van Boeckel

Historically, pharmaceutical industries have focussed on the discovery of compounds that target the protein products of genes. The intermediary product between gene and protein, consisting of RNA, has remained largely unexplored. Several drugs targeting the rRNA of bacteria have been, however, in clinical use for over half a century. One of these drug classes, the aminoglycoside antibiotics, also targets human rRNA, and have been developed as therapeutics for genetic disorders. Targeting at the RNA level is an economical approach to address non-drugable proteins and targets that have failed to give leads by hits in HTS, as it can build on biological knowledge gathered over years. RNA also offers entirely new opportunities for drug development, such as targeting of non-coding RNA sequences.

Guido J.R. Zaman*
Paul J.A. Michiels
Constant A.A. van Boeckel
N.V. Organon
Lead Discovery Unit
Oss P.O. Box 20
5340 BH OSS
The Netherlands
*tel: +31 412 661 043
fax: +31 412 663 508
e-mail:
guido.zaman@organon.com

▼ Although the RNA component of the ribosome is the target of numerous antibiotics, the idea that other structured RNAs could make good drug targets has reached the interest of pharmaceutical companies only within the past several years.

Why consider RNA as a drug target?

Current drug therapy with registered medicines targets only a few hundred endogenous targets, mainly receptors and enzymes [1]. Genomics has revealed many novel candidate drug targets. The total number of genes in the human genome (around 35,000) is much lower than anticipated a few years ago, and only two to three times higher than the number of genes in simpler organisms such as *Drosophila melanogaster* (13,601 genes) or *Caenorhabditis elegans* (18,424 genes) [2]. Functional genomics is used to evaluate the potential value of novel genes for drug discovery. Although target validation studies help to make decisions and to set priorities throughout the drug discovery process, they do not deliver drug candidates directly. Thus, there is good reason to cherish the well-established targets, and to try to address these 'old' targets in a novel way, particularly if no drugs for these targets are available.

Drug discovery in larger and most mid-sized pharmaceutical companies is a highly industrialized process, in which the chances of finding good drug leads is enhanced by screening high numbers of targets against large collections of synthetic chemical molecules. Generic technologies to assay members from all major drug target classes (e.g. receptors, kinases and ion channels) are available, and greatly speed up the process from gene-to-screen. This drug discovery factory is successful: a recent world-wide study involving 50 pharmaceutical and biotech companies reported that 43% of the targets evaluated in HTS deliver lead hits [3]. In this study, a lead is defined as a compound showing biologically relevant activity that correlates to the target. In addition, the compound must show evidence that a SAR can be built around it. Since the start of HTS in the early 1990s, 62 of these leads from HTS have been developed further to drug candidates that were actually tested in humans [3]. Half of these leads were directed against kinases, proteases or other enzymes, and 25% were directed against receptors [3]. The major drug target classes (i.e. receptors, kinases, enzymes, ion channels and transporters) cover only 17% of the human genome [2]. The remaining part encodes proteins that are not readily amenable to HTS (50%), such as structural proteins, or are genes with unknown functions or expressed sequence tags (ESTs) (33%) [2]. Thus, not only is a large part of the human genome unexplored in current drug discovery, research on the well-established targets will deliver few new drug candidates.

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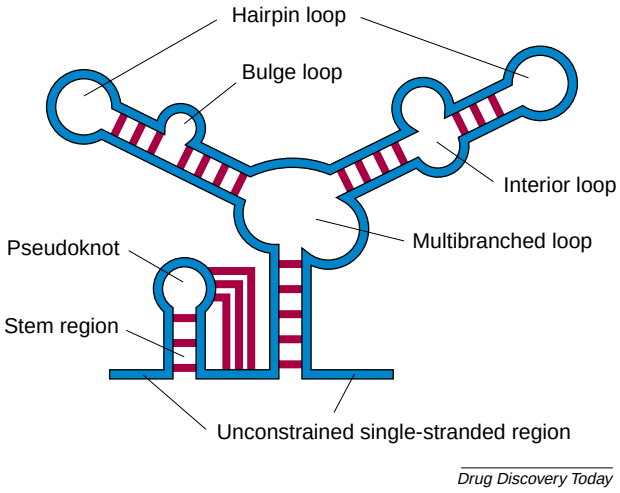

Figure 1. Possible secondary structures in RNA.

genes. Usually these compounds are agonists or antagonists of receptors, or compounds that inhibit or (less often) stimulate enzymes or protein–protein interactions. The intermediary product between gene and protein, consisting of mRNA, is largely unexplored. The development of RNA interference as a major platform for high-throughput target validation has put RNA in the spotlight of drug discovery research. RNA is not only used as a tool for target validation, but is also being developed as a therapeutic: RNA enzymes (ribozymes) targeting Her2 and VEGF receptor 1 are in phase I and phase II clinical trials for oncology (Herzyme and Angiozyme from Ribozyme Pharmaceuticals Inc.; http://www.rpi.com/index.html). An RNA aptamer directed to VEGF is in phase III for age-related macular degeneration (Macugen, Eyetech Pharmaceuticals Inc.; http://www.eyetk.com/). In addition, RNA itself is a potential target for small-molecule drugs.

A characteristic feature of RNA is that it can fold into complex three-dimensional structures comprising loops, bulges, pseudoknots and turns (Fig. 1). These structures are responsible for the diverse functions of RNA molecules within cells. In this respect, RNA resembles more a protein than DNA, which is less flexible and has a less diverse tertiary structure. The unique shapes in various target RNAs create potential binding sites for small molecules.

Compounds identified by screening RNA are completely different from those classes that have been picked up by screening proteins. Thus, when screening RNA, one may enter a completely different chemistry. This provides new challenges, but also new prospects for companies to build up IP.

RNA-targeting drugs: the aminoglycoside antibiotics

The successful therapeutic application of the aminoglycoside antibiotics for over half a century, has been appreciated in many reviews as a kind of proof-of-principle that RNA can function as a drug target [4–6]. Indeed, aminoglycosides act by binding to the RNA component of the small ribosomal subunit, the 16S rRNA [7–10]. Both the therapeutic effects and the side-effects of aminoglycoside antibiotics are determined by other factors as well, however. These factors and the precise mechanism of action of the aminoglycosides have been resolved only recently.

The term aminoglycosides refers to the kanamycin, gentamicin and neomycin families of antibiotics. The structures of these aminoglycosides are shown in Fig. 2a. Aminoglycosides exert their antimicrobial activity by inducing misreading of the genetic code. This results in the loss of functional proteins and the accumulation of abnormal proteins in the bacterial membrane [11]. This compromises the barrier function of the membrane and finally results in cell lysis [11].

Evidence for the involvement of the 16S rRNA in aminoglycoside action was provided by *in vitro* reconstitution experiments performed in the 1980s [12]. It was shown that aminoglycoside-resistant ribosomes could be constructed by combination of the ribosomal proteins of sensitive bacteria and the 16S rRNA of resistant bacterial strains. These resistant species, such as *Streptomyces* and *Micromonospora*, actually produced aminoglycosides. To prevent their own destruction, these aminoglycoside-producing bacteria express methylases that modify the 16S rRNA at specific bases in a region that is involved in the decoding of the mRNA (the decoding loop) (Fig. 2b). This region is located in the A-site of the 30S ribosomal subunit. It was shown that the expression of 16S rRNA mutants in *Escherichia coli* could confer resistance to aminoglycosides, and that resistance in cells correlated with a decreased binding of aminoglycosides to the 16S rRNA mutants [13].

The direct binding of aminoglycosides to 16S rRNA has been demonstrated by various techniques, including NMR [7], surface plasmon resonance [8], mass spectroscopy [9], and X-ray crystallography [10]. In competition experiments using fluorescent aminoglycosides, the affinities of the aminoglycosides for the 16S rRNA range from 0.1 μM to 10 μM, depending on the aminoglycoside structure used [8–10,14]. Binding is mediated through hydrogen bonding and electrostatic interactions between the phosphate backbone of the RNA and the positively charged amino groups of the aminoglycosides (Fig. 2c).

The same aminoglycosides also bind to the RNA of the small ribosomal subunit of many other prokaryotic and eukaryotic species, but do not bind to the 12S rRNA of human mitochondrial ribosomes [15–17]. However, certain individuals contain a point mutation in the 12S rRNA gene that causes aminoglycoside hypersensitivity [18]. The

12S rRNA mutant is now bound by aminoglycosides [17]. When individuals with this mutation are treated with normal, low doses of aminoglycosides, toxic side effects on hair cells of the inner ear may result in permanent loss of hearing. Such studies indicate that the binding of aminoglycosides is dependent on the size of an asymmetric interior loop [17]. This region consists of three nucleotides in *E. coli* 16S rRNA (Fig. 2b). In wild-type human mitochondrial 12S rRNA, this interior loop comprises seven nucleotides, whereas the point mutation associated with aminoglycoside hypersensitivity results in the formation of an additional base pair and reduces the size of the loop to five nucleotides (Fig. 2b).

Aminoglycoside binding differs only moderately between prokaryotic 16S and human 18S rRNA [15]. Nevertheless, aminoglycoside antibiotics only kill bacterial cells. This selective cytotoxicity has been explained by sequence differences [19] and by the occurrence in prokaryotes of transporter proteins that actively take up and concentrate aminoglycosides in the cytoplasm [11]. Cellular uptake mechanisms for aminoglycosides also exist in eukaryotic cells. In the human body, for example, aminoglycosides specifically accumulate in renal tubular epithelial cells and in hair cells of the inner ear, where also the undesired side-effects are observed. Mice deficient for megalin, a receptor of aminoglycosides that is expressed in the renal proximal tubuli, do not accumulate aminoglycosides in the kidney [20]. Thus, the expression and activity of cellular uptake mechanisms is an important factor determining the positive and negative biological effects of aminoglycosides, in addition to the binding to rRNA.

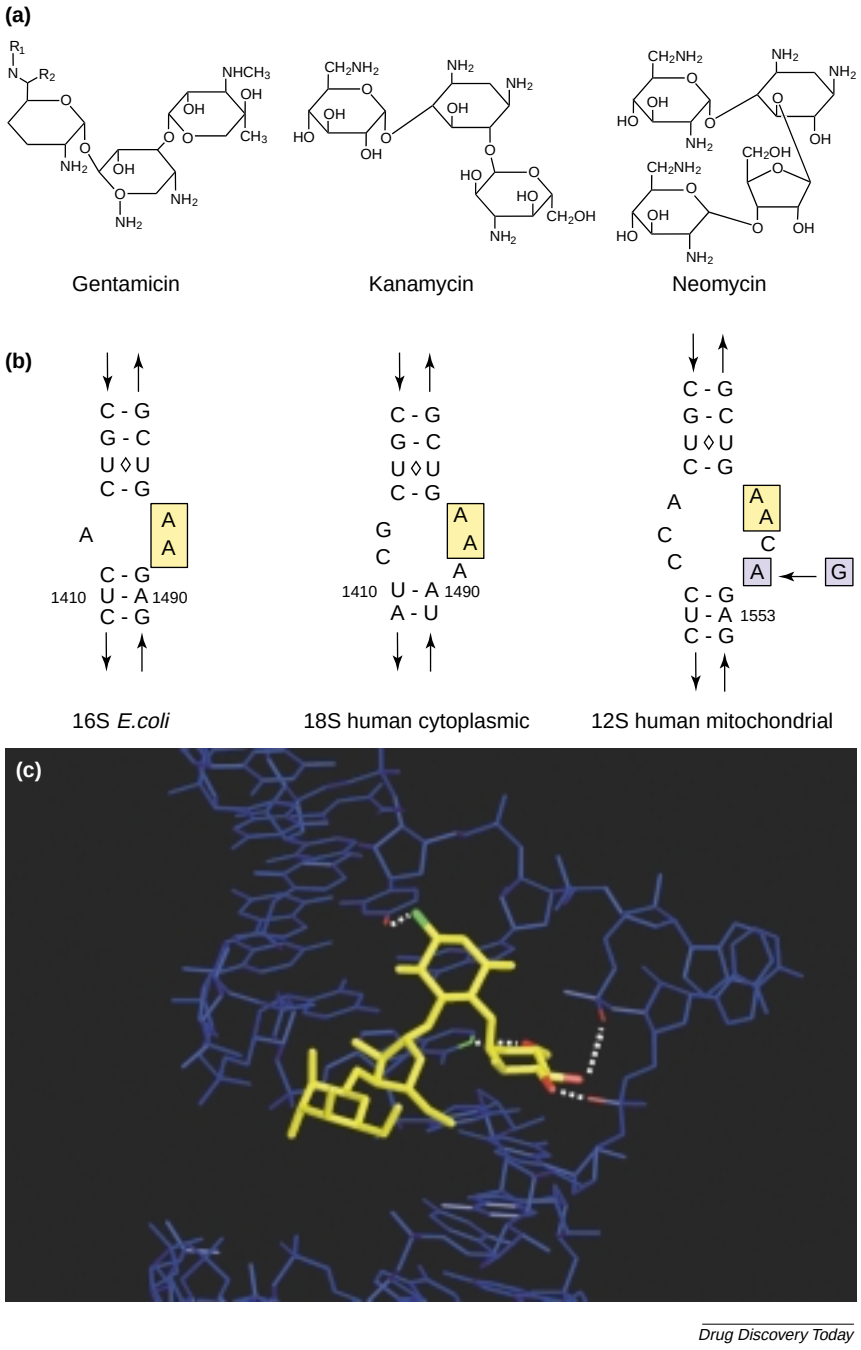

Figure 2. (a) Structures of the aminoglycosides gentamicin, kanamycin and neomycin. **(b)** Sequences of aminoglycoside target sites in rRNA species. The univer sally conserved adenine bases, located at positions 1492 and 1493 of the *E. coli* 16S are highlighted in yellow. Mutation of the adenine at position 1555 of the human mitochondrial 12S rRNA to guanine is associated with aminoglycoside hypersensitivity. This base and the substituent are highlighted in blue. **(c)** Model of the neomycin analogue paromomycin (shown in yellow) complexed with 16S rRNA (shown in blue), based on the X-ray crystal structure in [61] (picture created with the program MOLMOL [62]).

A model to explain the induction of translational misreading by aminoglycosides has been inferred from the crystal structure of the 30S ribosomal subunit in complex with paromomycin [10] (Fig. 2c). Critical in this model are two universally conserved adenine residues, located at positions 1492 and 1493 in the bacterial rRNA (Fig. 2b). In the process of decoding of the mRNA, A1492 and A1493 are flipped out

from the A-site to interact with the codon–anticodon duplex formed between aminoacyl-tRNA and mRNA. Paromomycin binding alters the structure of the rRNA so that the two bases are flipped out already. This conformational change induced by paromomycin reduces the energy required for binding of both cognate (correct) and non-cognate (incorrect) transfer RNAs, thus resulting in an increased error rate of the ribosome. It should be noted that the NMR structure of a 16S rRNA A-site fragment with paromomycin did not reveal the full conformational change that the antibiotic imposes on rRNA upon binding [7]. This shows the importance of the ribosomal context for aminoglycoside action.

rRNA and aminoglycosides in drug discovery today

Bacterial rRNA is a target that is still being explored in drug discovery today. Research sponsored by the U.S. Defense Advanced Research Projects Agency (DARPA) is ongoing to design novel rRNA-targeting drugs that are active against a broad spectrum of pathogenic bacteria [21]. These drugs may be used in the defense of bioterrorism attacks. To look for new targets for broad-spectrum antibiotics, the rRNA genes of many bacteria are searched for conserved secondary structures. It is assumed that the more frequently a predicted structure is found across pathogenic bacterial species, the more likely it is real, and the better it should be as a drug target for a broad-spectrum antibiotic.

The human cytoplasmic 18S rRNA is also being explored as a target for drug discovery. The antibiotics G-418 and gentamicin have been shown to cause read-through of a premature stop codon in the gene for the cystic fibrosis transmembrane conductance regulator (CFTR), a transmembrane chloride transporter [22,23]. These premature stop codons in the *CFTR* gene occur in a 5% subpopulation of cystic fibrosis patients. Read-through resulted in increased levels of full-length CFTR protein and restored CFTR-mediated chloride transporter activity in a CF bronchial epithelial cell line [23]. There were no immediate detrimental effects on other cellular functions. A few CF patients have even been treated with aminoglycosides and an improvement of CFTR-mediated chloride transporter function was reported [24]. The suppression of stop codons is also being explored as a potential novel treatment for Duchenne muscular dystrophy (DMD), which is caused by the absence of the dystrophin protein in striated muscle [25], and Hurler syndrome, a lysosomal storage disease [26]. In DMD, 6% of all cases arise from premature stop codons in the *dystrophin* gene [25]. Promising *in vivo* data were obtained with a mouse model for DMD in which the function of dystrophin was restored by treatment with gentamicin [25].

The identification of heterocyclic compounds with biological properties similar to aminoglycosides in causing translational read-through was recently reported by PTC Therapeutics Inc. (http//www.ptcbio.com) [27]. The compounds were identified by HTS using a luciferase reporter assay that was applied in cells and in *in vitro* translation extracts. The compounds were more active than the aminoglycoside gentamicin in inducing translational read-through. One of the compounds restored the biochemical function of CFTR in an ion channel assay, and relieved symptoms of DMD in a mouse model.

The mechanism underlying translational read-through is not fully understood. Read-through induced by aminoglycosides is determined by the sequence of the stop codon and other surrounding sequences, in particular the nucleotide immediately downstream from the stop codon [28]. In an *in vitro* system, high concentrations of aminoglycosides induced read-through up to a level of 63% of all translation reactions [28].

Targeting of viral RNA

Besides rRNA, aminoglycosides can bind in a saturable fashion and with similar micromolar affinities to a variety of other RNA structures containing non-duplex elements, such as bulges or interior loops. Aminoglycosides can bind to the Rev protein response element (RRE) in the *envelope* gene of HIV-1 [29] (Fig. 3a). RRE is the binding site of the viral Rev protein and plays a role in the transport of unspliced RNA from the nucleus to the cytoplasm. Inhibition of Rev–RRE interaction by aminoglycosides blocks the production of HIV-1 in tissue culture [29]. The transactivation-responsive element (TAR) is another stem-loop structure in HIV-1 RNA that is bound by aminoglycosides [30] (Fig. 3a). TAR is a substrate of the viral Tat protein and is essential for viral replication.

Besides the aminoglycosides, a large variety of synthetic molecules, including heterocycles, polycations and intercalators, have been found to bind to RNA specifically [5]. Various potent inhibitors of the interaction of HIV-1 Tat with TAR have been described, some showing inhibition of growth of the virus in tissue culture [31,32].

Targeting of mRNA

Potential target sites in mRNAs

Clearly, drug discovery on mRNA has only just begun. It is likely that tertiary structures in mRNA, such as hairpins or pseudoknots, will provide more selective binding sites for small molecules than stable double stranded domains, or the non-structured AU-rich elements (AREs) that have been mapped in the 3′ untranslated regions of various mRNAs coding for cytokines, oncoproteins and growth factors [6]. These AREs are bound by a family of RNA-binding proteins that regulate mRNA translation and stability. The individual members of this family bind with different affinities to

different ARE-containing mRNAs, thus providing some selectivity. Additional selectivity originates from the differential expression, both in time and 'space' (i.e. cell type/tissue) of the RNA-binding proteins and cognate mRNAs. Some of these interactions have critical functions in physiological processes, such as the fine-tuning of inflammatory reactions, and are explored as targets in drug discovery [6].

Although the structure of mRNA is considered to be less complex than that of rRNA, there are a few well-defined secondary structure elements in mRNA described in literature. One is the iron response element (IRE), which is contained in several mRNAs involved in iron homeostasis (Fig. 3b) [33]. At low cytosolic concentrations of iron, the IRE is bound by the iron sensor protein aconitase. When the concentration of iron in the cell increases, the protein dissociates from the IRE. In some mRNAs the IRE is located in the 5′ leader, where it functions as a translational silencer. In other mRNAs, IREs are located in the 3′ untranslated region and function to increase the stability of the mRNA. Recently, an IRE was identified in the 5′ leader of the Alzheimer's amyloid precursor protein [34], and is considered as a small molecule drug target [35].

Another example of a well-defined secondary structure in mRNA is the autoregulatory stem-loop structure in the 5′ end of the thymidylate synthase mRNA (Fig. 3a) [36]. Thymidylate synthase regulates its own synthesis by binding to and stabilizing this stem-loop structure, which includes the AUG start codon. Aminoglycosides have been shown to bind to this stem-loop structure [36]. Thymidylate synthase is an important target enzyme in cancer chemotherapy and an example of an established drug target that is being re-addressed at the mRNA level.

Preferably, the mRNA structures and mechanisms targeted should be unique and specific. Various mRNAs contain internal ribosome entry sites (IRESs) that are activated under conditions of stress, such as γ-irradiation, hypoxia or amino acid starvation. IRES elements by-pass the need of the 5′ cap the ribosome uses to scan to the translation initiation site of an mRNA. IRES elements have been proposed as drug targets, on the basis of the dramatic physiological and pathological consequences associated with the disturbance of IRES function [37]. For example, a point mutation in an IRES located in the *c-myc* mRNA correlated with increased c-Myc translation in cell lines from patients with multiple myeloma [38]. Such observations suggest that small changes in IRES structure can have a profound effect on protein expression. Small molecules targeting these sites may also have profound effects. However, as the protein factors involved in IRES-mediated translation act on many mRNAs, and since IRES elements are thought to share some common shape, it may be difficult to obtain selectivity.

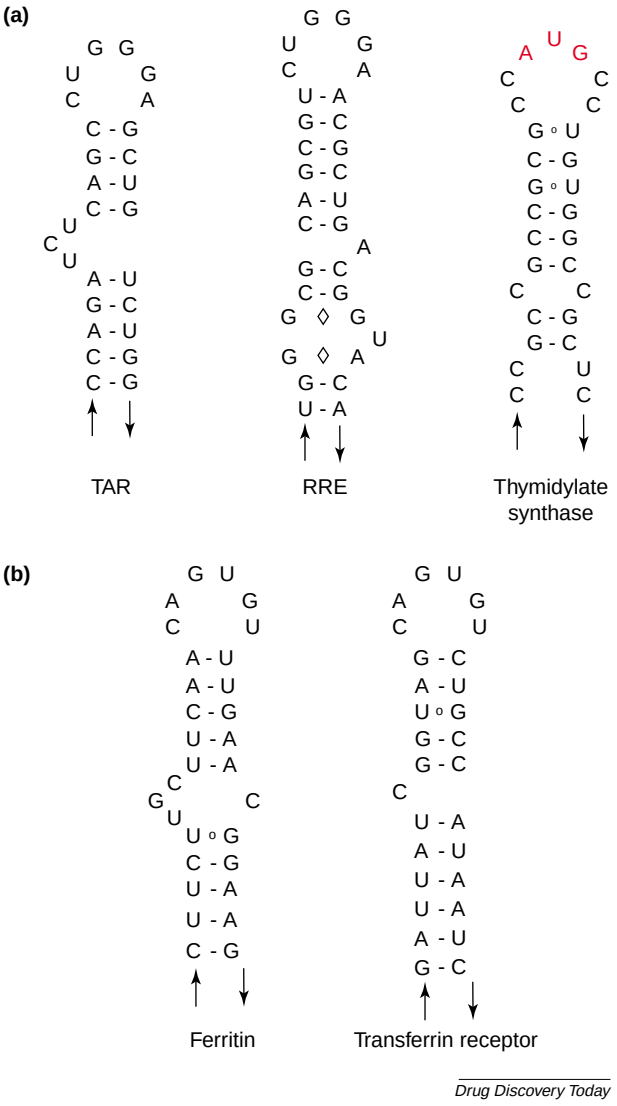

Figure 3. (a) Target sites of aminoglycosides in eukaryotic viral and cellular mRNAs. The stem-loop structure in the thymidylate synthase mRNA includes the start codon, which is indicated in red. **(b)** IREs in the 5′ leader of ferritin mRNA and 3′ untranslated region of transferrin receptor mRNA. Ferritin is an iron-storage protein. The transferrin receptor is involved in the cellular uptake of iron. At low cytosolic concentrations of iron, aconitase binds to the IRE in both the ferritin and the transferrin receptor mRNA. This results in the inhibition of ferritin translation. At the same time, the stability, and consequently the translation, of the transferrin receptor mRNA is increased. In response to increased levels of iron, aconitase dissociates from both mRNAs. This results in increased synthesis of ferritin and iron storage, while the synthesis of the transferrin receptor and the cellular uptake of iron are decreased.

Role of gene regulation at the mRNA level

An understanding of why gene regulation at the mRNA level has evolved (or been maintained during evolution), can help in the identification of new mRNA targets and the

prediction of the biological effects of compounds directed against those targets.

In energetic terms, post-transcriptional regulation is an expensive mechanism to control the expression of genes. The mRNA is only an intermediate in the multistep process from gene to active protein. If a cell would regulate this process only at the beginning, at the transcriptional level, it would save the energy needed to accommodate, degrade and recycle the mRNA molecules that are not used to synthesize proteins. Indeed, in prokaryotes, lacking a nuclear membrane and the separation in space of transcription and translation, examples of regulation at the RNA level are scarce.

Regulation of cytokine gene expression through AREs in cells of the immune system provides a mechanism to fine-tune a biological response. Regulation of various cellular RNAs through IRES elements provides a rapid response. Reticulocytes (immature red blood cells) lack a nucleus. Therefore, there is an obvious need for control of gene expression at the mRNA level. Regulation at the mRNA level is also a common control mechanism during embryonic development, oogenesis and spermatogenesis.

Spermatogenesis

Towards the end of spermatogenesis, the spermatid nucleus is highly condensed and transcriptionally inactive. At these stages, all regulation of gene expression occurs at the post-transcriptional level. Several examples of RNA targets and RNA-binding proteins involved in spermatogenesis are known, and are potential targets for the discovery of new male contraceptives.

Approximately 10% of males who are infertile due to the absence of sperm production (i.e. who are 'azoospermic') contain deletions in a Y chromosomal region encoding the RNA-binding protein DAZ (for deleted in azoospermia) [39]. Genetic studies in mice and *Drosophila* of an autosomal DAZ-like gene (DAZL) support a critical role of DAZ/DAZL in the production of germ cells [40–42]. Recently, the target of DAZL on mRNAs involved in spermatogenesis has been identified [43].

The TAR RNA-binding protein was originally identified by its ability to activate HIV-1 TAR-dependent translation [44], and it is expressed in testis [45]. The murine homologue, *PRBP*, binds to a structured element in the 3′-untranslated region of *protamine-1* mRNA [46]. Protamines mediate the compaction of nuclear DNA during spermatogenesis. After synthesis, the protamine mRNAs are stored as translationally silent mRNA ribonucleoprotein particles for several days. Deletion of the gene coding for PRBP, or its target in the *protamine-1* gene from the murine genome, results in impaired protamine translation and male sterility [47,48]. The human homologue TRBP is likely to play a similar critical role in male spermatogenesis [45].

The search for novel targets in mRNA

The above overview provides a few examples of defined structures and regions in human mRNA, of which some are potential drug targets. The search for new mRNA targets goes on. Whereas genomic comparison is a powerful tool to identify target sites in prokaryotic rRNA, the genomic information of higher eukaryotic species is still too limited to be used for the identification of targets in human mRNAs. This limitation holds in particular for the sequences of the 3′ untranslated regions of the mRNAs, as these regions show less conservation than the protein-coding part.

RNA-binding proteins may be used as a tool to identify new RNA targets. In the near-completed sequence of the human genome, more than 300 copies of the evolutionary conserved RNA recognition motif (RRM) have been identified [2]. In addition, there are two other major classes of eukaryotic RNA-binding domains: the double-stranded RNA binding domain (dsRBD) and the K-homology (KH) domain [49]. RNA-binding domains may be used to precipitate mRNAs with regulatory sequence elements from cell extracts.

Sequences involved in regulation can be identified by functional studies and narrowed down by deletion analysis. To predict the secondary structure of these sequences, there are established computer programs such as mFOLD [50]. These programs utilize free energy minimization protocols and are considered to yield good results, especially for shorter sequences. However, beyond its secondary structure, RNA can form a large variety of complex three-dimensional shapes. It is expected that the recent elucidation of several X-ray and NMR structures of RNA and RNA–protein complexes will aid in the development of new tools to predict RNA tertiary structure [51]. Apart from these bioinformatic tools, chemical and enzymatic probes are available to investigate RNA folding and to map interaction sites with proteins and small molecules [52].

Drug discovery technologies for targeting of RNA

The studies on the aminoglycoside antibiotics outline how the interaction of small molecules with RNA can be investigated. The employed methods, such as NMR and X-ray crystallography, are generally low throughput. Researchers at IBIS Therapeutics (http://www.ibisrna.com) have developed a method based on electrospray mass spectroscopy, enabling the screening of tens of thousands of compounds a day [9,53]. A major advantage of this method is that no tags are needed to detect the binding of compounds to RNA. However, because an expensive high-resolution mass

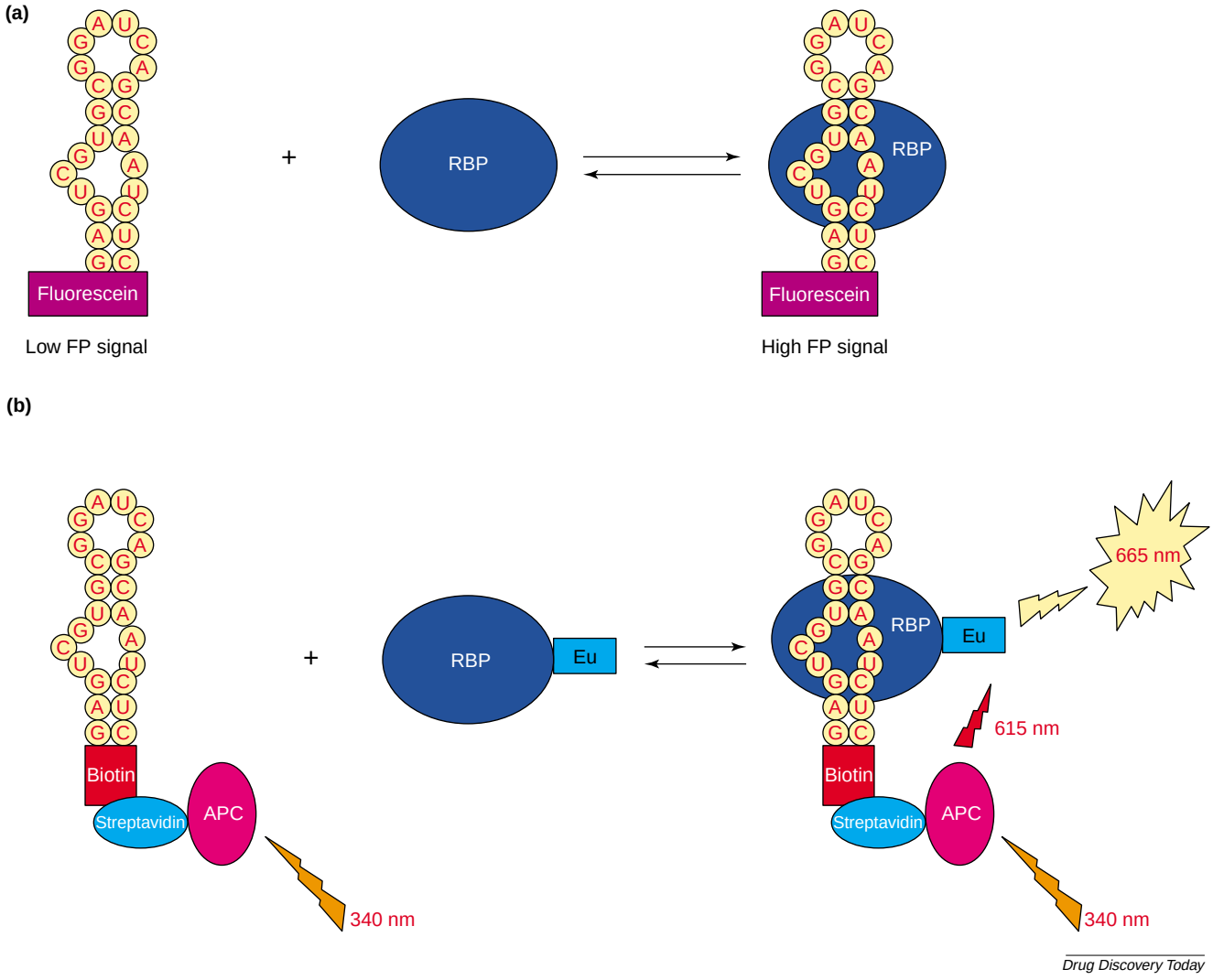

Figure 4. Principle for protein–RNA binding assay with **(a)** fluorescence polarization (FP), or **(b)** time-resolved fluorescence resonance energy transfer (TR-FRET) read out. (a) Fluorescein-labeled RNA and purified RNA binding protein (RBP) are mixed to form a protein–RNA complex. The FP signal increases when the relatively small fluorescent RNA molecule is bound by the relatively large RBP. A small molecule inhibitor of the protein–RNA interaction decreases the FP signal. (b) RNA labeled with allophycocyanin (APC) and RNA-binding protein (RBP) labeled with europium (Eu) are mixed to form a protein–RNA complex. Proximity of the donor (APC) and the acceptor (Eu) probes results in TR-FRET. A small molecule inhibitor of the protein–RNA interaction decreases the TR-FRET signal.

spectrometer is required, this method is not easily transferable to other laboratories. Surface plasmon resonance (SPR) is another technique by which the binding of small molecules to RNA can be monitored directly. In almost all these studies, RNA is biotinylated after synthesis and bound to a streptavidin-coated chip. SPR is very sensitive and easy to use and, with current equipment such as the Biacore 3000, the binding of 100 compounds a day can be measured. The recently launched Biacore S51 is able to handle even 1000 assays a day.

Other methods allow measurements of the binding of compounds or protein to RNA molecules in HTS. Some of these methods are based on the possibility of labelling RNA molecules with fluorogenic groups. The binding of RNA-binding proteins to these fluorescent RNAs can be measured by fluorescence polarization (FP) (Fig. 4a), or if both the RNA and the protein are labeled, by time-resolved fluorescence resonance energy transfer (TR-FRET) (Fig. 4b). FP and TR-FRET are preferred read-out technologies for biochemical assays in HTS [54]. Both technologies are robust, sensitive and versatile. Other advantages are that assays can be set up in a homogenous format (i.e. requiring no separation or washing steps, mix-and-measure), and do not involve radioactivity.

Scintillation proximity assay (SPA) is another screening method, which, to its disadvantage, does apply radioactivity. SPA has been used for the identification of inhibitors of Tat-TAR interaction [55]. In these studies, a short peptide derived from the basic Tat RNA-binding domain was used. The short peptide shows similar TAR-binding characteristics as full-length Tat. The use of peptides instead of proteins greatly facilitates assay development and eliminates the need for the expression and purification of large amounts of recombinant proteins, which is probably the most limiting and expensive step in HTS.

There are also methods that monitor the binding of small molecules to RNA in the absence of RNA-binding proteins. The binding of compounds that are coupled to beads (i.e. compounds synthesized by combinatorial chemistry) to fluorescent RNAs can be measured by fluorescence polarization [56]. Or, if the binding of a small molecule changes the equilibrium between the folded and unfolded state of an RNA stem-loop structure, it can be measured using a labelled complementary oligonucleotide [57], or by fusion of the stem-loop structure to a ribozyme (allosteric ribozymes) [58].

In cells, the interaction of RNA-binding proteins with RNA can be measured using reporter gene fusions [55]. The configuration of such assays for screening and their robustness is strongly dependent upon the specific cell line used. There are several indications that regulatory mechanisms working at the post-transcriptional level can be dependent on cell line or tissue. It is also well established that many genes have tissue-specific splice variants. It will not always be easy to translate or maintain these mechanisms in assays suitable for HTS. Thus, although screening in a cellular environment removes the need to identify and purify all molecular components involved, it increases the risk of artefacts and failure.

Conclusion

Targeting mRNA is a challenging new approach that is complementary to traditional drug discovery focussing on proteins. Addressing at the RNA level well-established protein targets that have failed to give good leads in HTS is economical, as it does not require long and expensive functional genomic studies as with new targets, but can build on biological knowledge that has been gathered over many years. In addition, targeting mRNA creates new strategies for drug discovery, such as protein upregulation by increasing the stability of an mRNA. Furthermore, targets that are usually not considered for the discovery of small-molecule drugs, because they are neither enzymes nor receptors, can become amenable to HTS. This category includes many targets that have been identified by genetic linkage studies, but also ESTs showing interesting phenotypes in knockout mice, or by their tissue distribution.

Where next?

The studies with rRNA-targeting aminoglycosides and heterocyclic compounds targeting HIV-1 TAR show that the activities of RNA in cells can be modulated with small molecules. The mechanism underlying the translational misreading induced by aminoglycosides is well understood because of the resolution of the crystal structure of the ribosome. These studies show the importance of the protein (ribosome) context in the binding of small molecules to RNA by facilitating conformational changes. It should be realized, however, that the positive (therapeutic-) and negative (side-) effects of these prototype RNA-targeting drugs are not only due to their binding to RNA, but also are strongly influenced by the activity of receptors and transporter proteins that mediate the cellular uptake of aminoglycosides in prokaryotic and eukaryotic cells. These observations underscore the importance of the biological environment of the targeted RNAs, when studying RNA-targeting compounds.

Potential target sites in mRNA molecules, in particular the binding sites of proteins regulating mRNA translation or stability, have been identified. Information on the structure and folding of RNA molecules (the 'ribonome') is increasingly becoming available through X-ray and NMR studies. Methodologies to screen for small compounds targeting RNA have been developed and are accessible, as they are based on the same principles and read-out technologies that are applied routinely for drug discovery on protein targets.

It may be noted that the modulation of endogenous, cellular mRNAs by small-molecule drug leads has not been demonstrated yet. In two recent articles in *Chemistry and Biology* [59] and *Nature* [60], it was shown that vitamins bind to bacterial mRNAs to control the translation of genes involved in vitamin metabolism. These so-called 'riboswitches' are potential targets for the development of novel bacterial anti-metabolites. We think that similar regulatory mechanisms involving the binding of small molecules to RNA stem-loop structures may exist in eukaryotic cells. If riboswitches also occur in human cells, additional opportunities for drug discovery would be created.

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