Drugs Made of RNA: Development and Application of Engineered RNAs for Gene Therapy

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Abstract: During the past decade, RNA has become a focus of investigation into new therapeutic schemes: antisense RNA, interfering RNA and *trans*-cleaving ribozymes are used to silence undesired gene expression. As an additional option with its own therapeutic potential, ribozymes may be employed to specifically alter the sequence of RNA. Among these RNA based strategies the mode of action varies: while antisense and interfering RNAs are capable of making specific contacts to other RNA molecules with the result of employing the cellular machinery for degradation of the RNA target, *trans*-cleaving ribozymes fold into specific three-dimensional structures to form catalytic centres and to specifically cleave a chosen RNA target. Beyond this, *trans*-splicing ribozymes have been engineered to first cleave a RNA target followed by ligation of a new RNA fragment delivered with the ribozyme. The latter strategy potentially extends the application of ribozymes from inhibition of gene expression to RNA repair, *i. e.* correction of genetic disorders at the level of RNA, and has already shown promising results in cell culture experiments. On the other side, advances in RNA synthesis, ribozyme engineering, delivery methods and expression systems have greatly enhanced the prospects of ribozymes, antisense and interfering RNAs in gene therapy.

This review provides an overview of existing strategies for potential RNA based gene therapy. It is focussed on the engineering of ribozymes and functional RNAs to be used as drugs and on the basic molecular principles of their action.

Key Words: Antisense oligonucleotide, gene therapy, RNA, RNA repair, ribozymes, siRNA.

INTRODUCTION

Over the past two decades our understanding of RNA has been revolutionised. Ribozymes have been discovered in nature and were developed in the test tube. The major importance and involvement of non-coding RNAs in processes of cellular regulation became clear and has developed into a major subject of research (reviewed in [1]). Furthermore, a number of riboswitches located in the non-translated leader region of messenger RNAs were found to be involved into regulation of gene expression [2]. These structural motives can directly bind small molecules that are often the final product of a metabolic reaction catalysed by the encoded protein and in this manner act as riboswitches for genetic regulation. The exact structure and the mechanism of functioning of more and more riboswitches is currently elucidated. RNA in its many facets is more and more understood and we are now in the position to design and use functional RNAs as valuable tools in molecular biology and medicine. Thus, within the exciting discoveries mentioned above, RNA has become a focus of investigations into novel therapeutic schemes. *Trans*-cleaving ribozymes, antisense RNAs and siRNAs have been used to silence undesired gene expression [3]. All three strategies have in common, that the synthesis of a protein that is pathogenic to the cell is inhibited at the level of the messenger RNA. Thus, antisense RNAs as well as *trans*-cleaving ribozymes are of considerable use for treatment of malignant or viral diseases. However, if one would think of the therapy of inherited diseases such as Cystic Fibrosis or Sickle Cell Anaemia, strategies that allow for correction of genetic disorders rather than for their destruction are required. "Repairing ribozymes" may be uniquely suited for this purpose, because compared to more traditional methods that attempt to correct a genetic deficiency by transferring a wild-type DNA version of a gene to the cell, ribozymes might repair incorrect transcripts without interfering with the corresponding DNA.

Thus, *trans*-splicing ribozymes as well as a number of other schemes have been investigated to demonstrate the potential of nucleic acid repair in functional genomics. For example, mobile group II introns, that are catalytic RNA structures capable of inserting directly into a chromosome and subsequently being reverse-transcribed into DNA, have been successfully used to target DNA sites in human cells [4]. Single-stranded triplex forming oligonucleotides (TFOs) have been applied to correct a mutation in plasmid DNA [5] as well as an adenosine deaminase (ADA) mutation in human lymphocytes, facilitating DNA repair of the mutation by the nucleotide excision repair pathway [6]. Furthermore, double-stranded oligonucleotides capped by hairpins and consisting of both DNA and RNA residues (so-called chimeras) have shown efficacy in correcting single-base genomic changes in mammalian, animal and plant cells [7-9].

Taken together, RNA is not only a valuable drug target, it can also be designed to act as drug itself. While on one side, RNA can be targeted with antisense oligonucleotides, ri-

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bozymes, proteins or small molecules to inhibit expression of a specific gene, on the other side, antisense RNAs, ribozymes, siRNAs or aptamers are designed to function as drugs. In the past years, we have seen an impressive development of new strategies to design, to deliver and to control the action of therapeutic RNAs, documented in a large number of papers. While individual areas have been reviewed separately, we here present an overview on the field concentrating on RNA drugs acting on RNA.

ANTISENSE RNA

Antisense oligonucleotides are used in the treatment of a variety of diseases, which are caused by the expression of deleterious genes, e.g. viral infections, cancer growth and inflammatory diseases. The small 13-25 nt single-stranded oligomers bind *via* Watson-Crick base pairing to their target and thus can potentially interfere with several steps of RNA processing and message translation including splicing, polyadenylation, export, stability and protein synthesis [10,11]. A disadvantage of the application of "naked" DNA- or RNA-like antisense oligonucleotides is their insufficient stability against nucleases. To increase nuclease resistance, one of the non bridging oxygen atoms of the phosphodiester backbone was replaced by sulfur, defining the "first generation" antisense oligonucleotides (Fig. (**1a**)). The thio modification can increase the half life of an antisense oligonucleotide to about 8-9 hours compared with unmodified DNA [12-14]. A disadvantage of phosphothioate oligonucleotides is their low specificity in target recognition as well as high toxicity [15].

"Second generation" antisense drugs show improved properties being less toxic and displaying slightly enhanced affinity towards their targets [14,16]. Typical features of these antisense oligonucleotides are 2'-O-alkyl modifications, such as for example 2´-O-methyl- or 2´-O- methoxyethyl- groups (2´-MOE) (Fig. (**1b**)).

Looking at "third generation" antisense oligonucleotides, one finds DNA or RNA analogues with modified linkages and/or with completely different chemical moieties replacing the sugar furanose ring (Fig. (**1c**)). These oligonucleotides show high affinity towards their targets, little toxicity and greatly enhanced stability against degradation by nucleases.

Even though, RNA antisense oligonucleotides have been designed and studied in the laboratory, the majority of drugs

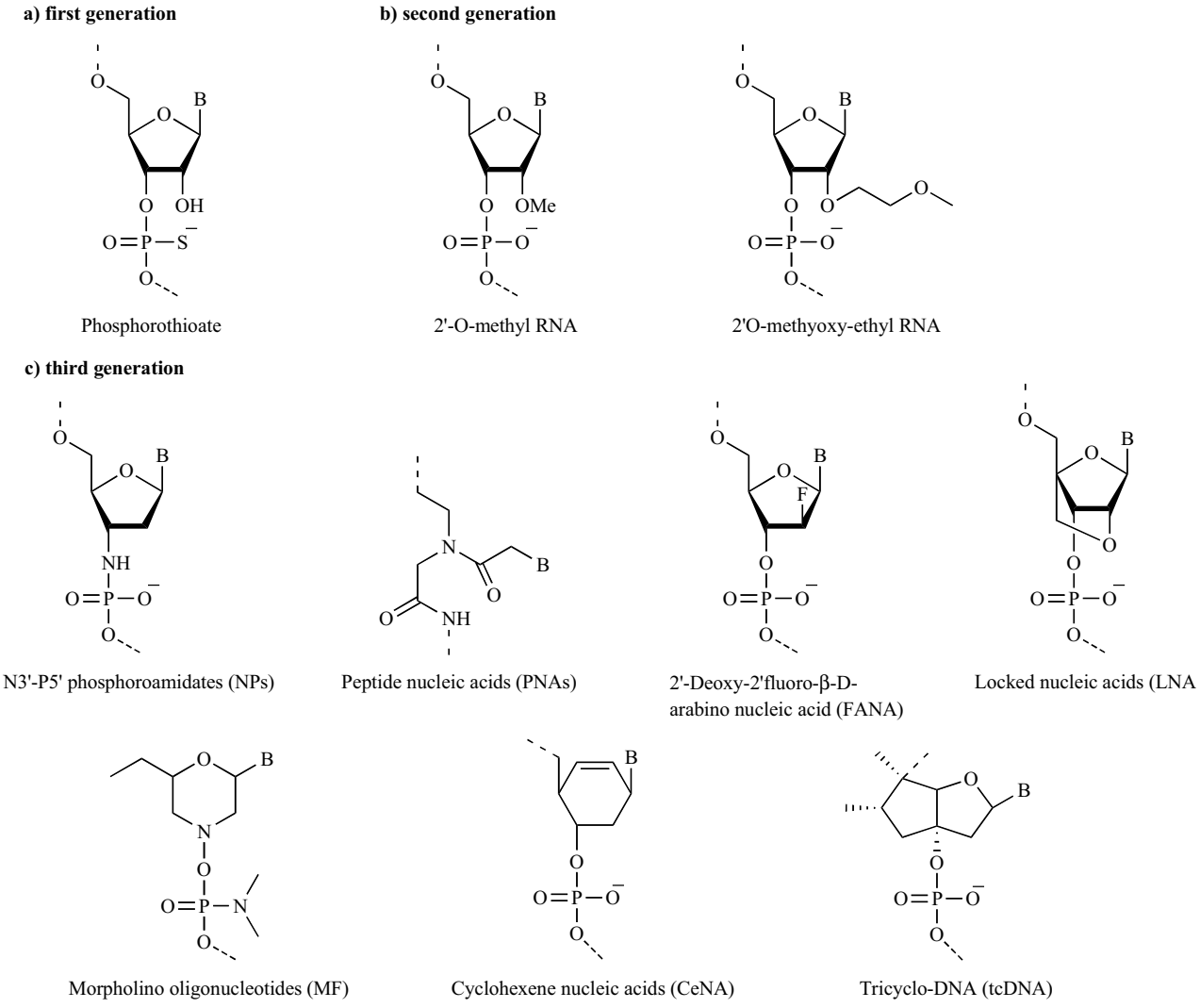

Fig. (1). Antisense oligonucleotides of the first (a), second (b) and third (c) generation.

in clinical research still consist of deoxyribonucleotide analogues functioning *via* an RNaseH dependent mechanism. Binding of the antisense nucleotide to its target results in the recruitment of RNaseH leading to degradation of the target RNA. Wu and co-workers [17] have identified human RNaseH1 as the responsible species for RNA degradation. Overexpression of human RNaseH1 was shown to increase the activity of several antisense oligonucleotides targeting specific RNAs in several human cell lines. On the other side, reduction of RNaseH1 levels does not completely inhibit antisense oligonucleotide activity, indicating that other yet unidentified RNase activities may be involved in the process.

On the contrary to DNA based antisense oligonucleotides, RNA based antisense oligonucleotides and "third generation" antisense drugs work as pure steric blockers. These oligonucleotides physically prevent or inhibit progression of the splicing or the translational machinery. Dependent on the target region, translation initiation [18] or elongation can be prevented. In general, the coding region of a mRNA appears to be a less suitable target, due to the ability of the ribosomal machinery to resolve double-stranded structures and thus to unwind the antisense oligonucleotide from its mRNA target.

A number of antisense oligonucleotides that are working by these mechanisms are now being evaluated in clinical studies against diabetes, cardiovascular diseases, cancers, viral infections, asthma and others (see also http://www.isispharm.com/; http://www.avibio.com/devNeugene.html).

For example, antisense oligonucleotides are used in the treatment of diabetes. Type 2 diabetes is the most common form of diabetes. Even though producing insulin, the bodies of diabetes 2 patients do not react to insulin, hence glucose is not absorbed into cells but left in the blood stream. The protein tyrosine phosphatase 1B (PTP-1B) is an enzyme that appears to reduce insulin's ability to regulate blood sugar levels. For example, enhanced expression of PTP-1B was observed in animal models of diabetes as well as in patients with diabetes and insulin resistance [19]. Furthermore, polymorphisms that are associated with type II diabetes have been detected in the PTP-1B gene [20,21]. Mice in which the gene of PTP-1B was down regulated display enhanced sensitivity to insulin and resistance to obesity induced by a high fat diet [22]. Thus, inhibition of PTP-1B may help to keep the insulin receptors active for a longer time, allowing for more glucose uptake into cells and in turn for lower levels of glucose in the blood stream. Currently, Isis Pharmaceutics is implementing clinical studies with a second-generation antisense oligonucleotide, termed ISIS 113715, targeting PTP-1B in patients with type 2 diabetes. This drug was well tolerated in a Phase I study and did not cause hypoglycaemia, or excessively low blood sugar, the latter being an adverse effect observed with many currently available treatments for type 2 diabetes. ISIS 113715 is now being evaluated for a Phase II clinical study (see also http://www.isispharm.com/).

ISIS 369645 is an inhaled second-generation antisense drug that inhibits the alpha subunit of the interleukin 4 receptor, IL4R-α. Down regulation of IL4R-α production results in inhibition of two important cytokines IL4 and IL13, involved in the regulation of inflammation, mucus overproduction and airway hyper responsiveness. ISIS 369645 is one of the first antisense drugs to be developed for uptake by inhalation. Respirable antisense oligonucleotides (rAsONs) targeting discordantly expressed mediators of inflammation and/or bronchoconstriction being delivered to the lung *via* inhalation represent a new class of epigenetic-based therapeutics for asthma and other pulmonary diseases. The properties of these agents (solubility, chemical stability, rapid design based on primary DNA sequence information) combine synergistically with characteristics of the lung (non-invasive route of administration directly to the target organ, presence of uptake-modifying surfactant) to enhance the therapeutic potential of these oligonucleotide-based drugs. Their potential is further increased by the possibility of engineering antisense oligonucleotides, the effects of which are limited to the lung, reducing or avoiding the possibility of systemic toxicity. In an asthma mouse model, it was shown that an antisense inhibitor of IL4R-α potentially reduces the level of ILR4-α encoding mRNA as well as of the corresponding protein, along with reduction of cytokine production, inflammation and airway hyper responsiveness. The drug, if delivered by inhalation, was shown to rapidly distribute to the airways and to achieve therapeutic drug concentrations in multiple cell types with little systemic exposure. (http://www.isispharm.com/).

Antisense oligonucleotides can also mediate protein expression by modulating the splicing pathway. In this case, the ratio of different splice variants can be altered, such that consequently the function of a gene is varied. Here, it is important to note that about 60% of human genes are alternatively spliced and close to 50% of genetic disorders are caused by mutations that cause defects in pre-mRNA splicing (reviewed in ref. [23]). Therefore, antisense oligonucleotides that alter mRNA splicing in a strictly controlled fashion may have far-reaching implications in the treatment of a variety of diseases. Aberrant splice sites generated by mutations can be silenced, thus restoring correct splicing and function of the transcript of a defective gene. There are two major requirements for oligonucleotides that shift splicing: i) The drugs must not activate RNaseH, which would destroy the mRNA before splicing and ii) they have to be able to effectively compete with natural splicing factors. Members of the second and third generation antisense oligonucleotides fit these criteria.

There are three examples in which way antisense oligonucleotides can mediate the splicing procedure: i) blocking an aberrant splice site, which is caused by a mutation, and thus activating the correct splice site (Fig. (**2a**)), ii) blocking an intact splice site in order to skip an exon that contains a mutation (Fig. (**2b**)) or iii) targeting an appropriate splice site that would lead to translation of an alterative protein (Fig. (**2c**)).

Aberrant splice sites created by mutations can be found in several forms of the blood disorder β-Thalassemia. β-Thalassemia results from a reduced or absent functional β-globin that is a subunit of the haemoglobin. The defect decreases the oxygen-carrying capacity of red blood cells and causes compensatory expansion of the bone marrow. If untreated, this anaemia results in early death. For example, mutations at position 654 (C to T transition), 705 or 745 (C to G transversion) in Intron 2 of human *β-globin* pre-mRNA

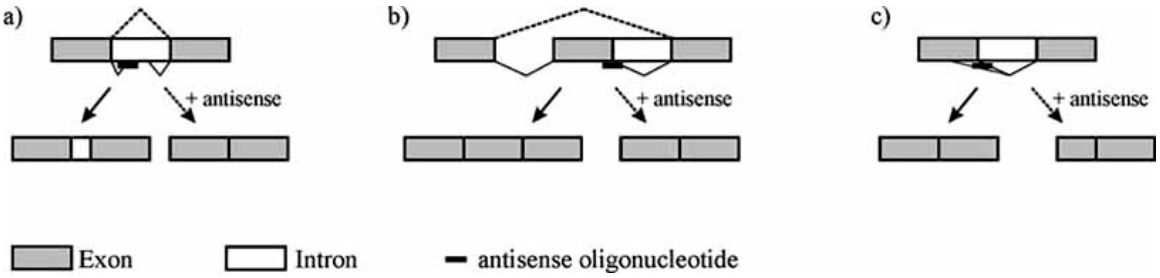

Fig. (2). Modification of splicing by antisense oligonucleotides.

lead to creation of an aberrant splice site within the intron Fig. (**2a**). This results in the inclusion of a part of the intron into the spliced out exon, thus creating an in-frame stop codon [24]. Sequence specific correction (about 30 % - 50 % for IVS2-654- and about 30 % for IVS2-745 erythroid cells compared to control oligonucleotides) of splicing and up regulation of *β-globin* with morpholino oligonucleotides was shown in human erythroid progenitor cells from thalassemia patients [25]. High levels of correct RNA and haemoglobin expression were achieved after several days of treatment [26,27] with exogenously delivered oligonucleotides. In an alternative and potentially longer-term strategy, the antisense sequences were incorporated into lentiviral expression vectors using an U7 promotor. These constructs have been used to treat human erythropoietic progenitor cells successfully *ex vivo* [28].

Another example for antisense oligonucleotides interfering with splicing pathways is exon skipping. This strategy has been used in treatment of Duchenne muscular dystrophy (DMD), a muscle degenerative disorder mainly caused by nonsense or frame-shift mutations in the dystrophin gene [29]. The human dystrophin gene is comprised of 79 exons. Deletion of exons from the area of exon 17 to exon 49 is associated with mild clinical phenotype [30], whereas deletion of exons in the area from exon 18 to exon 59 retains production of highly functional proteins [31]. The majority of DMD mutations occur within this latter, noncritical region of the gene, mostly creating stop codons resulting in translation of truncated proteins. Thus, skipping the mutated exon in this region or the exon(s) whose omission would restore the correct reading frame, can result in production of the protein retaining critical functions. In a mdx mouse model, 2′-O-methyl oligonucleotides targeting the 5′-splice site of exon 23 containing an inherited stop codon, were shown to induce skipping of this exon and consequently restoring the correct reading frame. As a result, the treated muscles produce dystrophin at near-normal levels. Furthermore, repeated administration of the antisense drug was shown to enhance dystrophin expression without eliciting immune responses [32,33]. A phase I clinical study for the treatment of DMD with antisense oligonucleotides is scheduled to start soon (see www.clinicaltrials.gov).

An example of directing alternative splicing by antisense oligonucleotides as shown in Fig. (**2c**) is targeting the bcl-x transcript associated with the development of cancer. The gene *bcl-x* encodes two splice variants with opposite functions that are derived from the use of two alternative 5′-splice sites. The long form, bcl-xL, has antiapoptotic properties, while the short form, bcl-xS, has proapoptotic properties. Both forms are required for the normal cellular functions. However, bcl-xL is overexpressed in a number of cancers, including prostate cancer [34]. Blocking the xL 5′-splice site should increase the ratio of bcl-xS to bcl-xL and thereby simultaneously increase proapoptotic signals and decrease antiapoptotic signals, thus causing the death of a cancer cell or at least inhibiting its growth. Taylor *et al.* targeted the sequence adjacent to the xL splice site with 2′-methoxyethyl phosphorothioate oligonucleotides delivered by cationic lipids to A549 lung carcinoma cells [35]. A dose-dependent and sequence-specific increase in bcl-xS expression was seen, with optimal shifting occurring after 24 hours and with 100-200 nM oligonucleotide. The newly synthesised bcl-xS was successfully translated into protein. However, the increased bcl-xS level was not effective in inducing cell death. Instead, A549 cells were additionally sensitised to apoptosis by *cis*-platinum. Interestingly, Mercatante *et al.* demonstrated that an antisense 2′-O-methyl oligonucleotide targeted to the xL splice site alone induced apoptosis in PC3 prostate cancer cells [36,37]. Here maximal shifting of splicing occurred with 80 nM oligonucleotide approximately 16-24 hours after delivery by cationic lipid transfection. Villemaire and co-workers used another interesting approach to modulate bcl-x pre-mRNA splicing [38]. A 2′-O-methyl oligonucleotide was designed being partially complementary to an upstream region of bcl-xL and carrying a 5′-tail with two high affinity binding sites for hnRNP A1. Thus, the antisense oligonucleotide positions the A1 protein in the vicinity of a 5′-splice site and impedes splicing by a steric blockade. As a result, splicing was shifted toward bcl-xS. Presumably, this effect is caused by interference with splice site recognition or with spliceosome assembly. The investigated oligonucleotide showed higher activity in four different cell lines compared with duplex-forming oligonucleotides against sequences upstream of the 5′ -splice site as well as with oligonucleotides directly complementary to the 5′-splice site itself. Thus, oligonucleotides complementary to exogenic sequences may improve the specificity of action, whereas maximal interference with splice site recognition is conferred by the hnRNP A1/A2-binding tail. Using such antisense oligonucleotide/hnRNP A1/A2 heteromers is a high potential application for the treatment of cancers, because these proteins are abundant in growing cells.

Each of the second and third generation oligonucleotides displays excellent binding affinity and resistance to degradation. However, none of the RNA-like oligonucleotides support significant RNaseH activity, neither do peptide nucleic acids, methylphosphonates or morpholino backbones. Fortu-

nately, this limitation can be overcome by the use of chimeric oligonucleotides, so called gapmers. These oligomers usually consist of a central stretch of deoxynucleotides or phosphorothioate deoxynucleotides and modified nucleotides such as 2′-O-methyl ribonucleotides at each end. The modified nucleotides at both termini prevent nucleolytic degradation and the contiguous stretch of four to eight deoxy residues (or more) between flanking 2′-O-methyl nucleotides was reported to be sufficient for activation of *E. coli*- and human RNaseH [16,39,40]. Gapmers have shown high affinity to their RNA target and low protein binding, thus leading to enhanced potency and reduced toxicity.

Clusterin, a protein that is associated with resistance to apoptosis, has been found to be overexpressed in androgen-independent prostate cancer, in non-small cell lung cancer, bladder cancer, and other tumor types. Targeting clusterin with an 21-gapmer (13 deoxynucleotides in the centre, flanked by four 2′-O-methoxyethyl nucleotides at each end) was found to enhance castration- and chemotherapy-induced apoptosis in prostate cancer xenografts [41]. The mRNA level of clusterin was decreased in human PC-3 tumor cells in a dose-dependent and sequence-specific manner. The gapmer ISIS 112989/OGX-011, directed against clusterin, is currently in Phase 1 clinical trials. Further antisense drugs being in clinical trial are summarised in Table **1**.

TRANS-CLEAVING RIBOZYMES

Another strategy for inhibition of gene expression involves cleavage of mRNAs by ribozymes. Ribozymes are ribonucleic acid molecules with enzymatic activity. They are capable of sequence-specific cleavage of suitable RNA substrates. With respect to the substrates they act upon and to the products they produce, this characteristic feature makes them potent molecules for therapeutic applications [42,43]. Ribozymes cleave the rather unreactive bonds of a phosphodiester linkage in an RNA molecule resulting in a 3′-hydroxyl group and 5′ phosphate or in a 5′-hydroxyl group and a 2′3′-cyclic phosphate [44]. To the group of so-called small ribozymes belong the hammerhead (HH), hairpin (HP), varkud satellite (VS) and hepatitis delta virus (HD) ribozymes [45] Fig. (**3**). The first hammerhead ribozyme was identified in the virusoid from Lucerne transient streak virus in 1987 [46]. Hairpin ribozymes were first derived from the "minus" strand of the satellite RNA of tobacco ringspot virus [47]. HD ribozymes were discovered in genomic and antigenomic RNAs of the hepatitis delta virus (HDV) [48] and the largest of the small ribozymes, the VS ribozyme, was originally found in mitochondria of *Neurospora* [49].

The reaction these RNAs catalyse is a simple nonhydrolytic cleavage whereby the scissile bond undergoes a nucleophilic attack by the adjacent 2′-OH group. The reaction produces 2′3′-cylic phosphate and 5′-OH termini (Fig. (**4**)) [50]. Whereas the VS ribozyme is a metalloenzyme requiring divalent metal cations for catalysis [51], the HP ribozyme does not necessarily need divalent metal ions as catalytic co-factor [52]. The role of divalent metal cations for the catalytic reactions of HH and the HD ribozymes has not been thoroughly solved. In the case of hammerheads, the mechanism of catalysis has been postulated to involve single [53], double [54] and no divalent metal cations [55].

The HH, HP, HD, and *Neurospora* VS ribozymes have all been converted from the naturally occurring *cis*-active ribozymes to *trans*-active ribozymes by splitting the catalytic core from the substrate sequence. The *Neurospora* VS ribozyme has been converted once and is the least understood motif [56]. More work has been performed on the *trans*-active HD ribozyme. As the secondary and tertiary structure of this catalytic RNA motif is not as well studied as the HP and the HH motifs, it was more difficult to design *trans*-active HD ribozymes [57,58].

Nonetheless, *trans*-active HD ribozymes have been developed [59,60]. As already mentioned the development of the HH and HP catalytic domains for *trans*-cleavage are far more advanced. The structures and RNA folding are fairly well understood and the models for the design of *trans*-acting hammerhead and hairpin ribozymes are well tried and tested. The hammerhead (Fig. (**3**)) consists of two flanking arms capable of base pairing with the substrate to form two helices (I and III) and a catalytic core with a helix (II) and

Table 1. Antisense Oligonucleotide Drugs in Clinical Development

Antisense Drug	Target	Disease	Clinical Trials	Type of Oligonucleotide
ISIS 301012	ApoB-100	elevated cholesterol	Phase 2	2nd generation
LY 2181308(ISIS 23722)	Survivin	cancer	Phase 1	gapmer
ATL 1102 (ISIS 107248)	VLA-4	multiple sclerosis	Phase 2	2nd generation
ISIS 104838	TNF-α	rheumatoid arthritis	Phase 2	gapmer
Resten-NG	c-myc	restenosis	Phase 2	3rd generation
GEM640	XIAP	Refractory or relapsed acute myeloid leukemia	Phase 1	2nd generation
MG98	DNA Methyltransferase 1	Metastatic retinal carcinoma	Phase 2	2nd generation
GEM 231	Protein kinase A	Cancer	Phase 1	2nd generation
LY2181308 (ISIS 23722)	Survivin	Cancer	Phase 1	2nd generation

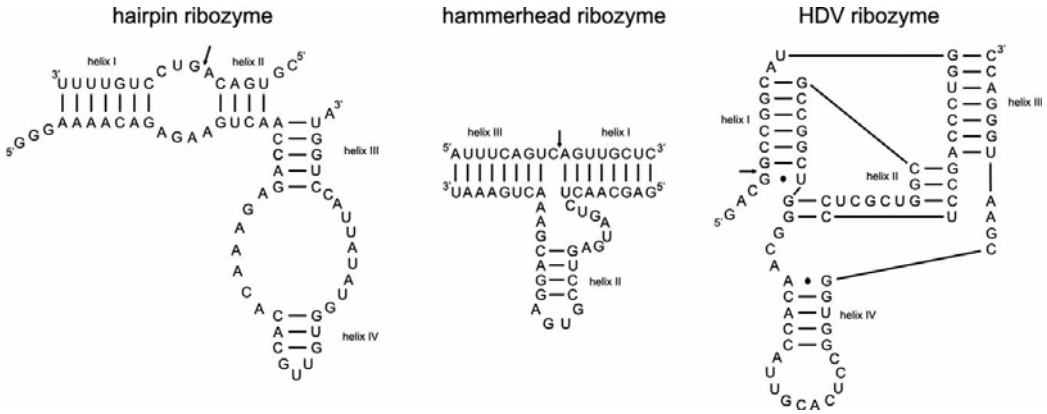

Fig. (3). Exemplary catalytic RNAs. Arrows mark the position of scissile bonds.

several single-stranded regions. The ribozyme recognises sequences at either side of a substrate NUH (N: A, C, G, U; H: A, C, U) site by means of the flanking arms. The catalytic core then cleaves the RNA 3′ of the NUH triplet [61].

The hairpin ribozyme (Fig. (**3**)) has also been split into substrate and catalytic core [62]. It consists usually of 50 to 70 nucleotides in size. The secondary structure consists of four helices separated by two internal loop sequences.

All ribozymes are active *in vitro,* in cell-free systems and in living cells. This activity and their sequence specificity should make them all attractive as agents for the inhibition of gene expression. But as HD and VS ribozyme motifs sequence requirements for effective cleavage are rather demanding, research focused on hairpin and even more so on hammerhead ribozymes in search of therapeutic applications.

Challenges for design and application of catalytic RNAs as therapeutics are efficient entry into cells, ribozyme stability, and precise targeting to the substrate RNA sequences. Availability of divalent cations and pH in cells must also be taken into consideration. The intracellular Mg^{2+} concentration is known to be approximately 1 mmol/L, much lower than is commonly used for *in vitro* reactions [63]. Nonetheless, as has been extensively reviewed recently [64-68] ribozymes have been used against oncogenes and viruses (HIV-1, HCV) [69] and have helped in understanding the role of genes in developmental processes.

There are two ways of delivering ribozymes to their targets: Exogenous delivery: presynthesised ribozymes are taken up by the target cells, and endogenous delivery: plasmids encoding the ribozyme are introduced into the nucleus where the ribozyme is transcribed and transported into the cytoplasm [70,71].

Unprotected RNA molecules are susceptible to degradation by 3′-exonucleases and pyrimidine-specific endonucleases in human serum. To stabilise ribozymes, mainly modifications of the phosphodiester linkage and the 2′-OH group of the ribose have been used (Fig. (**5**)) [72].

Ribozymes process their target RNA sequence by forming Watson-Crick base pairs and cleave it as described above. After dissociation of the cleaved RNA fragments they can process a new substrate (Fig. (**6**)).

There are different approaches to increase cleavage efficiency and target specificity of ribozymes, some more unusual than others.

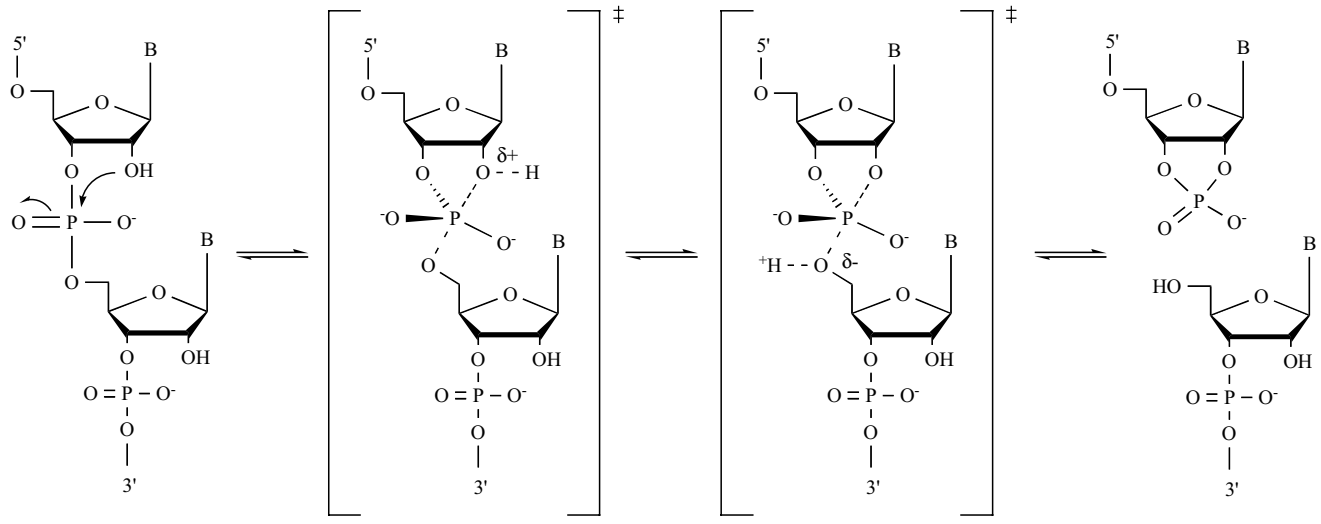

Fig. (4). Mechanism of the transesterification reaction catalysed by ribozymes. B: any nucleobase.

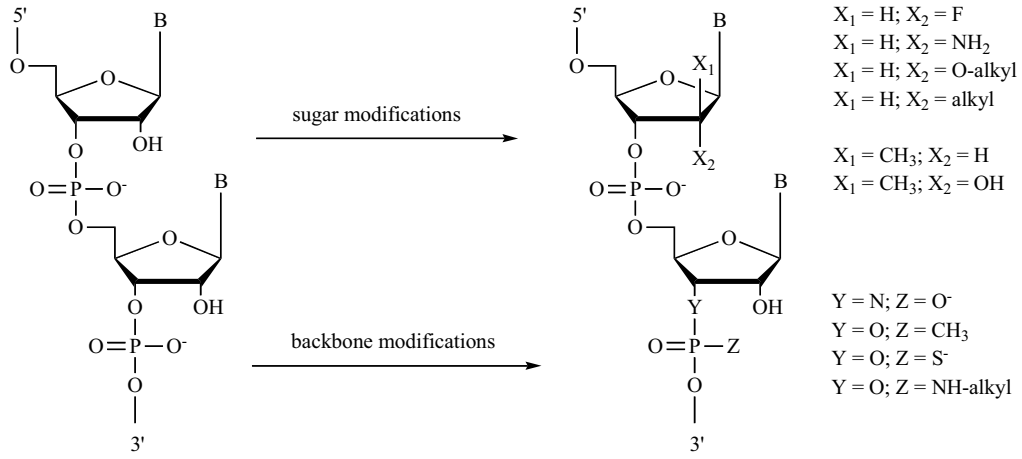

Fig. (5). Modifications to stabilize RNA. B: any nucleobase [73].

Taira and co-workers, *e.g.*, developed a new type of ribozyme, the so-called maxizyme [74]. The term "maxizyme" stands for minimized, active, x-shaped, intelligent ribozyme (Fig. (**7**)).

These ribozymes consist of two minizyme units (hammerhead ribozymes without stem loop II) [75-78], wherein one unit cleaves the target and the other unit serves as allosteric control. Whereas most minizymes showed quite low activity compared to the parental ribozyme, kinetic studies and NMR analyses revealed that this shortened ribozyme exhibited extremely high activity as dimer [79]. Taira and co-workers successfully tested maxizymes against abnormal chimeric *bcr abl* mRNA typical for CML (chronic myelogenous leukemia) *in vitro* and in animal models [80].

Chen *et al*., on the other hand, designed several mono-, di-, tetra-, penta- and nonaribozymes, so called multi-unit ribozymes, derived from the hammerhead motif, to target a highly conserved region of HIV-1 *env* RNA. The aim was to inhibit HIV-1 replication [81]. Leopold *et al*. targeted the *bcr-abl* mRNA in myeloid leukemias. Analysis of the mRNA showed the presence of three closely spaced NUH sequences. Instead of using three single hammerhead ribozymes they also designed a three-unit ribozymes consisting of three covalently bound hammerhead ribozymes (Fig. (**8**)).

Tests showed that this multi-unit ribozyme was more efficient in cleavage than three singly applied ribozymes [82].

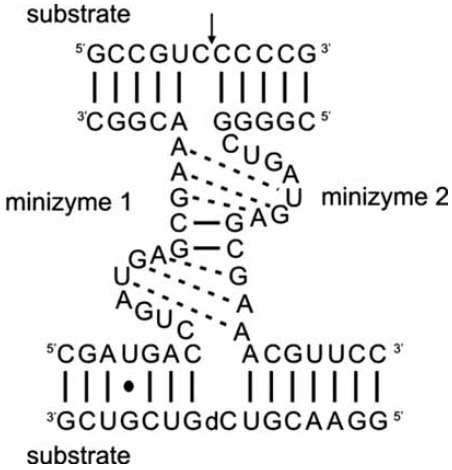

Fig. (7). Example of a maxizyme. The arrow marks the cleavage site.

Several Phase I and Phase II clinical trials are underway, where patients with either cancer or viral infectious diseases are treated with either gene therapy-mediated ribozymes

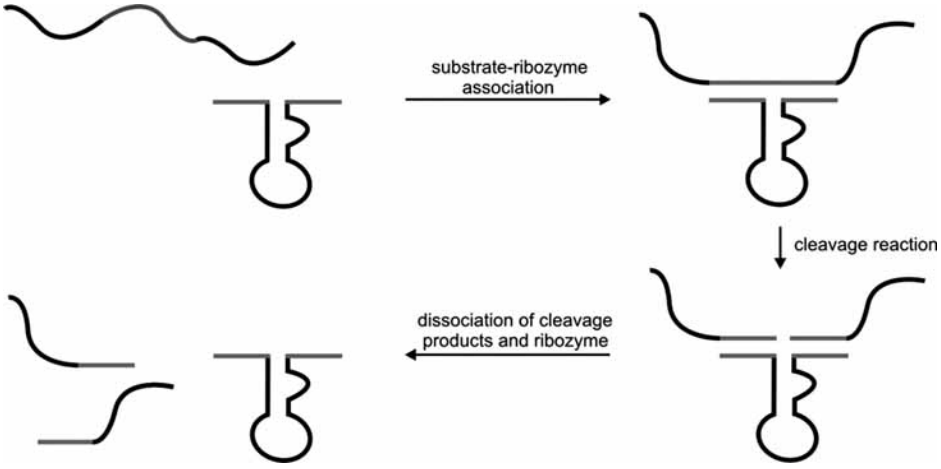

Fig. (6). Schematic presentation of ribozyme catalysed substrate cleavage.

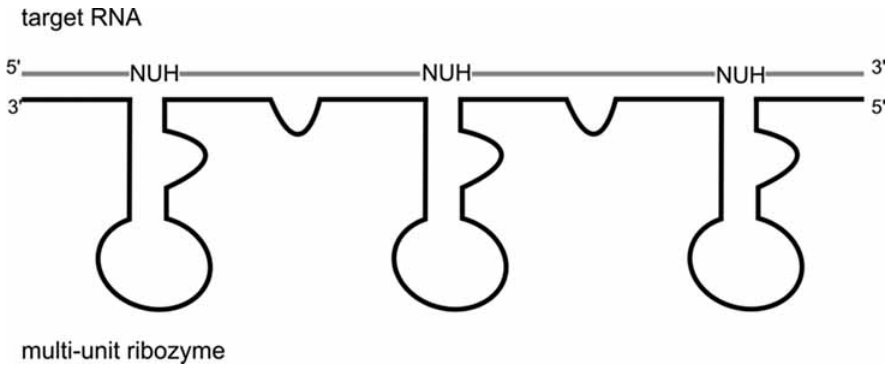

Fig. (8). Schematic illustration of a multi-unit hammerhead ribozyme.

(endogenous delivery), or direct injections of synthetically modified ribozymes (exogenous delivery). Gene therapy trials, mainly applied *trans*-cleaving hairpin ribozymes and hammerhead ribozymes directed against HIV-1 [83]. In such trials, CD4+ lymphocytes or CD34+ hematopoietic precursors from either infected subjects or uninfected identical twins are transduced *ex vivo* with retroviral vectors encoding the ribozyme [84-86]. However, transduced cells reached nearly undetectable levels within a year [87].

Other clinical trials rely on direct infusions of chemically modified ribozymes into patients. These trials have targeted an activated oncogene [88], a cellular receptor involved in angiogenesis in cancer [89], and Hepatitis B and C infections [90,91]. HERZYME, a synthetically modified 'zinzyme' targeting the human epidermal growth factor receptor type 2 (HER-2) mRNA, with growth inhibitory effects on breast, pancreatic, and ovarian cancer cells in culture and/or in mouse xenograft models [92-94], currently is being tested in a Phase I clinical trial with advanced, refractory cases of breast cancer. Preliminary results show stabilisation of disease, though without any partial or complete responses. ANGIOZYME, a hammerhead ribozyme, is directed against the mRNA for vascular endothelial growth factor receptor-1 (VEGFR-1). VEGFR-1 is a cell-surface receptor of endothelial cells and responds to VEGF, which almost all tumours produce [95]. Inhibiting VEGFR-1 prevents new blood vessels from growing such that tumour growth can be stopped. ANGIOZYME was initially documented to have anti-tumour, anti-angiogenic, and anti-metastatic effects in a xenograft model of metastatic lung cancer [89]. A Phase I/II clinical trial involving patients with advanced, refractory solid tumours completed in July 2001 documented that it was well-tolerated without significant side-effects. Later, ANGIOZYME entered Phase II clinical trials as monotherapy or in combination with standard chemotherapy for treatment of advanced breast or colorectal cancers. ANGIOZYME lowered serum levels of soluble VEGFR-1, but did not produce a significant clinical response, emphasising the importance of combining it with standard chemotherapy regimens. In the colorectal carcinoma trial, where ANGIOZYME was administered in combination with the Saltz regime, results have been encouraging: by 12 weeks, only 12.5% of patients on the combination therapy progressed, as compared to 25% of patients treated with chemotherapy alone.

HEPTAZYME, a synthetic ribozyme targeting the conserved 5′UTR of Hepatitis C viral RNA, is being tested in Phase II clinical trials of patients with chronic hepatitis C infection, who have an elevated risk of liver cirrhosis and hepatocellular carcinoma [72]. In Phase I/II clinical trials, HEPTAZYME was tolerated well, without significant toxicity; and in pre-clinical trials on cultured cells, combination therapy with type 1 interferon and HEPTAZYME reduced viral replication by greater than 85% while also significantly lowering the dose of interferon required to attain significant reduction in viral replication [96].

HerBzyme, the latest synthetic ribozyme targeting several Hepatitis B virus (HBV) transcripts as well as the HBV pregenomic RNA, is being tested in Phase I/II clinical trials [97]. In pre-clinical trials, it inhibited viral replication at several stages of the HBV life cycle, resulting in decreased HBV DNA, HBsAg, HBeAg levels in cultured cells, while significantly lowering viremia in transgenic mice [90].

In summary, ribozymes are promising tools for combustion of viral diseases and cancer. In cultured cells they have already proven to be functional. So far, only a few ribozymes have been tested in clinical trials and there are still a number of hurdles to be overcome before being capable of using more ribozymes as therapeutic tools. As for other RNA made drugs, the mobility of RNA inside cells, cellular factors that may impede ribozyme action, and of course, cell biology involving trafficking and intracellular localisation of RNA need to be investigated in order to develop efficient therapeutic strategies.

RNA INTERFERENCE

In addition to antisense oligonucleotides and ribozymes as described above, short double-stranded RNAs of 21-23 nucleotides in length are able to modulate the expression of complementary target mRNA, making them a potential drug in the treatment of many human diseases such as viral infections, tumours and metabolic disorders [98-102]. Based on their origin, these small RNAs can be grouped into two classes: small interfering RNAs (siRNAs) and micro RNAs (miRNAs). miRNAs are transcribed from endogenous genes by RNA polymerase II. The hairpins embedded in the primary structure transcripts of miRNA (pri-miRNA) are cropped into hairpin structured precursors (pre-miRNA) by a member of the nuclear endonuclease RNase III protein, termed Drosha. Pre-miRNAs are then exported from the nucleus by exportin-5 and subsequently processed into short mature miRNAs by a multidomain enzyme of the RNase III family called Dicer. Exogenous siRNAs are produced from a

long dsRNA also by the action of Dicer, which cleaves the dsRNA into short dsRNAs with 2-3 nucleotide 3′-overhangs [103-105]. Both the miRNA- as well as the siRNA- Dicer complex is associated with a dsRNA binding protein TRBP, albeit a second dsRNA binding protein called PACT is also associated with the miRNA-Dicer complex [106]. The double-stranded siRNA is loaded onto a multienzyme complex termed RNA induced silencing complex (RISC) which unwinds the double-stranded RNA. One of these strands, the guide strand, is recruited to the target mRNA to initiate degradation or translational repression of the target, while the other strand, the so-called passenger strand, becomes degraded. While perfectly complementary interactions between short RNA (siRNA or miRNA) and target mRNA result in mRNA degradation, mismatched interactions between siRNA or miRNA and target mRNA results in translational repression. The key effector protein of the human cleavable RISC is the human Argonaute protein 2 (hAgo2), which cleaves the phosphodiester bond of the passenger strand [107,108]. For standard siRNAs, passenger strand cleavage follows rapidly after Ago2 binding to the dsRNA. However, when cleavage is blocked by chemical modification or by mismatches between the siRNA guide and passenger strands, a slower "backup" pathway dissociates and destroys the passenger strand. After cleavage, the passenger strand becomes degraded while the guide strand is involved into maturation of the RNA induced silencing complex [107,109], which consists of DICER, the TRBP and Argonaute in the siRNA-RISC complex and additionally of the PACT protein in the miRNA-RISC complex at its minimal form [110]. The RNA-RISC complex interferes by Watson-Crick base pairing with a target mRNA and either cleaves the mRNA between the 10^{th} and 11^{th} nucleotide starting from the small RNA 5′-end or prevents its translation (Fig. (**9**)).

The Ago protein associates tightly with the siRNA [111-114] and is responsible for the "slicer" activity of the RISC complex [111,112,115]. Slicer activity has been mapped to the PIWI domain in Ago proteins through mutagenesis [111]. The secondary structure of the PIWI domain is similar to that of RNaseH or endonucleases V [111,116]. The 3′-end of the single-stranded siRNA is bound by the Argonautes PAZ domain [117,118]. Target binding and recognition by RISC is determined mainly by base-pairing between the 5′-portion of the siRNA. Crucial are ~9nt at the 5′-end of siRNA, because the target is cleaved between positions 10 and 11 from the 5′-end of a siRNA [119-123]. Dependent on the individual isoform of Ago proteins, the RISC complex has distinct functions (in humans about 8 Ago proteins were found, which are classified into two subfamilies: the PIWI subfamily and the eIF2C/AGO subfamily [124]), that are most probably determined by the PIWI domain of the Ago protein. Out of all known human Argonaute proteins (Ago1-4), only the human Ago2 is responsible for the "slicer" activity of the RISC complex. On the basis of the type of Ago protein that is recruited to the RISC, these complexes can be tentatively divided into two general types: a cleaving RISC and a noncleaving RISC. A cleaving RISC has dual functions that can direct the target mRNA either for cleavage or for translational repression, dependent on the base-pairing features between the small RNA and the target mRNA [119,125]. If the base-pairing complementarities between siRNA and its mRNA target is less extensive, the target mRNA might be physically unreachable by the active centre of the endonucleases (slicer) in the cleaving RISC [119,121, 126], resulting in a translational repression. Similarly, noncleaving RISCs lack endonuclease (slicer) activity of Ago proteins and can direct the target mRNA only for translational repression.

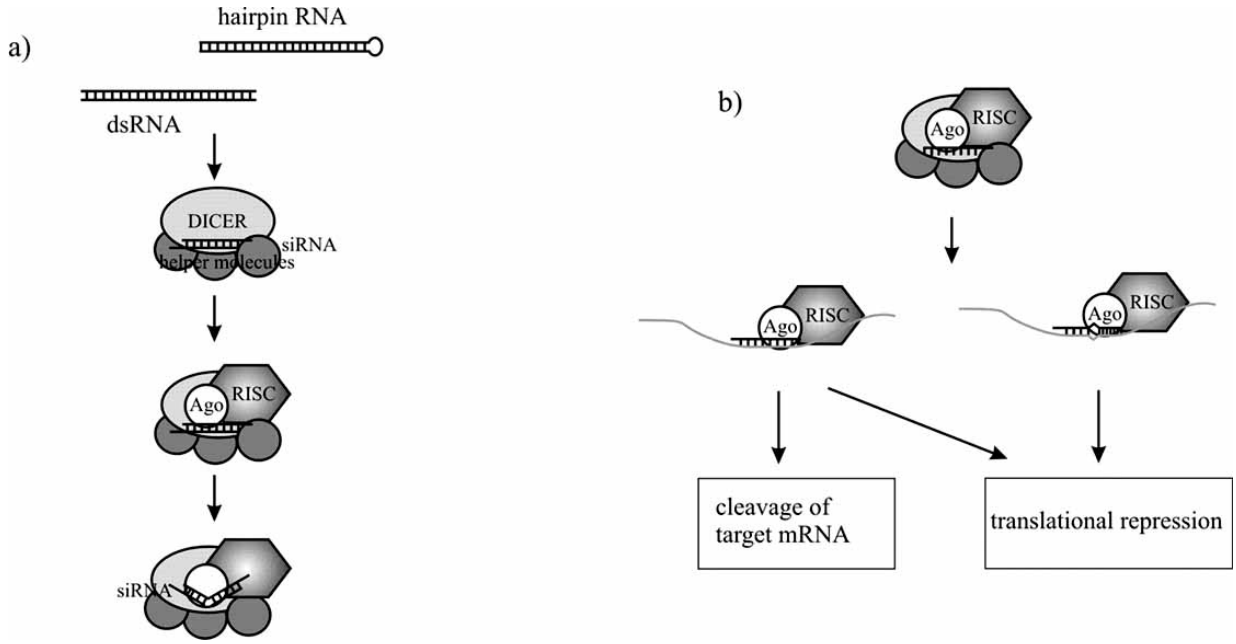

Fig. (9). Mechanism of a) siRNA processing and b) siRNA function. a) long double-stranded RNA or hairpin RNA is processed by a multienzyme complex, termed Dicer, into 21-23 short dsRNAs with 3′-overhangs, called siRNAs. After recruiting an Argonaute protein and RISC, the passenger strand is cleaved by human Ago2 and becomes degraded. b) The other strand, the so called guide strand, can bind to its target mRNA. Expression of the target mRNA can be inhibited by either hAgo2 initiated mRNA cleavage when the single-stranded siRNA is perfectly complementary to the target, or by a translational repression.

In addition to these two types of RISC, other kinds of RISC-like identity exist [127,128]. An example is the RNA-induced initiation of translational gene silencing (RITS) complex that directs chromatin remodelling. The RITS complex also contains DICER-generated siRNA and Ago protein, and functions in heterochromatic silencing by binding through the targeting promoter regions [129,130].

Using external double-stranded RNA for silencing a gene shows that long dsRNA of about hundred nucleotides in length induce an interferone response while short dsRNA of about 20 nucleotides induce RNAi in mammalian cells [131-134]. Long dsRNA activate a dsRNA-dependent kinase, the protein kinase R (PKR) [132,135] which phosphorylates and inactivates the translation factor eIF2α [135-137]. This results in inhibition of translation and in an altering cellular metabolism and often in activation of the JAK/STAT-pathway and global upregulation of interferon-stimulated genes leading to activation of apoptotic and non-apoptotic pathways [135,138,139]. A second long dsRNA-response pathway activates 2′,5′-oligoadenylate synthetase, the product of which is an essential cofactor for a sequence non-specific ribonuclease, RNaseL [135,137,138]. The activation of 2′,5′-oligoadenylate synthetase results in formation of 2′,5′-oligoadenylates that bind and activate RNaseL leading to inhibition of protein synthesis [140]. Furthermore, siRNAs do not produce toxic metabolites, have a high sequence specificity against their mRNA target, and induce RNAi at low concentrations [141,142], thus making them a preferred drug for therapeutic application.

Recent investigations however, have shown that siRNA-mediated silencing may be less specific than it was originally believed. Microarray profiling experiments have demonstrated siRNA-mediated silencing of numerous unintended (off-target) transcripts [143-145]. Sequence analysis of several off-target transcripts revealed only partial complementarities with the transfected siRNA, notably at the 5′- end of the siRNA guide strand [143]. These off-target silencing is widespread and occurs in a manner reminiscent of target silencing by miRNA [146]. The siRNA induced toxicity is concentration and sequence dependent; particularly on nucleotide positions 2-7 of the siRNA. Sequence variation may prevent the off-target effect of one transcript but can induce another. Position-specific and sequence independent chemical modifications that reduced silencing of partially complementary transcripts without affecting the on-target silencing have been described. siRNA with altering 2′-O-methyl and unmodified nucleotides [147], or altering 2′-O-methyl and 2′-O-fluoro nucleotides [148] displayed activity equivalent to unmodified duplexes, while full 2′-O-methyl modifications on the sense strand, antisense strand or both strands were inactive in transcript silencing [122,126,148,149,150]. However, a single 2′-O-methyl ribosyl substitution at position 2 of the siRNAs guide strand was shown to be sufficient to reduce off-target effects without affecting the knock down of the wanted target [151].

Immunostimulatory RNA motifs are more effectively recognised by innate immunity in the context of single-stranded siRNA as compared to siRNA duplexes [152]. Many sequences have been shown to induce interferone phenotypes through Toll-like receptor recognition, as for example GU-rich sequence elements GUCCUUCAA and UGUGU [153,154]. But also single-stranded siRNAs with low GU-content or without GU residues may activate the immune system. It is likely that other characteristics such as RNA structure, base position and base composition of flanking sequences may contribute to immune recognition and signalling. Replacement of uridines with adenines or of the 2′-OH group with either 2′-fluoro, 2′-deoxy or 2′-O-methyl uridines were shown to abrogate or reduce immune activation in human peripheral blood mononuclear cells [111].

A problem in using siRNA for therapeutic applications is their cellular stability. Relying on the result that several modifications increase RNA stability in antisense RNA, investigators have used this approach to stabilise siRNA. Initially, fully 2′-O-methyl modified sense strands of siRNA were shown to be inactive [122,126,147,149,150]. In contrast to these reports however, a fully 2′-O-Me modified sense strand against the tumour suppressor PTEN was shown to be active, displaying even comparable activity to the unmodified version of the sense strand [155]. These oppositional results show that the activity of chemically modified siRNAs may depend on their specific structure and sequence. 2′-substitutions at the 5′-end of the antisense strand tend to reduce potency, possibly dependent on the steric bulk of the substituent [156]. An exception to this general rule are alternating 2′-O-Me motifs with either 2'-OH [147] or 2′-fluoro groups [148]; those were shown to improve both stability and potency in certain cases.

Substitution of the oxygen in the ribose ring with sulfur has also shown good results [157]. Using siRNAs with a minimum number of thioribose modified residues resulted in good activity but brought only a small benefit in stability towards nucleases. The introduction of 4′-thioribose nucleotides at both termini of the siRNA strands increased the serum stability in HeLa cells 14-fold, while a combination of 4′-thioribose and 2′-alkylmodifications (2′-OMe, 2′-MOE) (see also Fig. (**1**)) was not only 13-18-fold more stable than unmodified siRNAs but also more potent. Also 2′-deoxy-2′-fluoro-β-D-arabinonucleotide (FANA) modified siRNAs show a higher nuclease resistance. This modification is compatible with the RNAi machinery and mediates specific degradation of target mRNA with increased activity. A fully modified sense FANA strand when hybridised to an antisense RNA was shown to be 4-fold more potent and had a longer half-life in serum (~6 h) compared with an unmodified siRNA (~15 min) [158]. The incorporation of FANA units is well tolerated throughout the sense strand of the duplex, but these modification can only be included at the 5′- or 3′- end of the antisense strand. The phenomenon that modifications are more tolerated in the sense strand than in the antisense strand was also shown using 2′,5′-linked nucleic acids [159]. The reason for the sensitivity of the antisense strand against modifications could be a change in geometry, making the antisense strand less suitable to interaction with the RISC complex.

Many studies have demonstrated the potential of RNAi to effectively silence the expression of viral genes and thus inhibit viral replication in cell culture or in mice. This approach has been applied to human immunodeficiency virus [160-163], rotavirus [164], dengue virus [165], influenza

virus [166], Hepatitis B- and C virus. Gene silencing can occur at several steps of infections: i) inhibition of the expression of viral antigens leading to blocking of virus entry, ii) transcription silencing, iii) blocking of viral replication, iv) silencing of viral accessory genes, v) hindrance of the assembly of viral particles and vi) inhibition of virus-host interactions.

A number of cellular membrane molecules may act as receptors for viruses. Silencing these receptors is also a suitable strategy to prevent the host from virus entry. In a HeLa derived cell line, targeting of CD4 as the main receptor for HIV-1, showed an 8-fold decrease of CD4 expression and a 4-fold reduction in viral entry [161]. A similar phenomenon was observed when targeting the human chemokine receptor protein CCR5, which is a necessary co-receptor for infection by most strains of HIV-1. Delivery of siRNA against CCR5 into human peripheral blood T lymphocytes resulted in a 10-fold inhibition of CCR5 synthesis on the cell surface over a period of 2 weeks and protected lymphocytes from HIV-1 virus infection with a decrease in infected cells by 3- to 7-fold [167]. The viral life cycle can be also interrupted at the transcription level of the viral genome. For RNA viruses, particularly retroviruses, *gag*, *env* and *pol* genes are essential for genome transcription. In several studies siRNAs were designed against HIV-1 *gag*, *pol* and *env* regions. The siRNAs were delivered prior to HIV-1 infection either by transfection [168,169] or using a lentiviral vector [170]. In all cases more than 90 % inhibition of HIV-1 production was observed.

The Hepatitis B virus (HBV) genome is a double-stranded DNA molecule that generates four viral RNAs encoding the core protein HBeAg as well as the polymerase-reverse transcriptase, HBsAg and the X protein. Using short hairpin (sh) RNAs targeting the core protein resulted in reduction of the protein level in Huh7 cells that contained a plasmid encoding a complete HBV genome [171]. A greater level of suppression was observed using siRNA that targeted both X and core mRNAs and not the core mRNA alone (79% *vs*. 63%), emphasising the potential synergistic effect of targeting multiple viral transcripts simultaneously. The authors further demonstrated the siRNA-mediated reduction of viral transcripts and reported a reduction in HBV replicative intermediates of up to 95%. Several studies have demonstrated that siRNA can efficiently inhibit HBV replication in animals. Although mice are not susceptible to HBV infections, the viral genome can be stably introduced to produce HBV transgenic animals that synthesise viral mRNAs in hepatocytes and secrete mature virions. Co-injection of a cloned HBV sequence with a plasmid expressing shRNA against distinct viral mRNAs, resulted in an effective RNAi response against HBV replication in mice [172]. Targeting a variety of sequences in each of the four viral mRNAs caused a reduction of up to 84.5 % in the level of secreted HBsAg in mouse serum and a higher than 99% reduction in the number of hepatocytes positive for HBcAg. Hepatitis and other liver diseases are ideal systems for RNAi because the liver is the primary organ for siRNA uptake [173,174].

As another feature, viruses have many accessory genes. These genes take part in the viral pathogenesis such as latency and persistence and regulate the expression of other genes. Therefore, silencing these genes should be a good therapeutic strategy for the treatment of viral diseases. The Epstein-Barr virus is related to the pathogenesis of several malignancies, including Burkitt´s and T-cell lymphomas, Hodgkin´s disease, breast and gastric carcinomas and some AIDS-related lymphomas. The EBV encoded oncoprotein, latent membrane protein -1, plays an essential role in cell transformation as well as in nasophyngeal carcinoma (NPC) metastasis. Targeting the LMP-1 mRNA with shRNA significantly altered EBV-positive NPC cell motility, substratum adhesion, and transmembrane invasion ability [175].

RNAi treatment of viral diseases has been used *in vitro*, *in vivo* and in some cases also in animal models with promising results. Though, RNAi technology is not limited to the treatment of viral diseases. The RNAi strategy was also used in a cellular model of frontotemporal dementia with parkinsonism [176], in a mouse model of Alzheimer´s disease [177], cancers and age related macular degeneration (AMD). The macula is located near the centre of the retina at the back of the eye. It provides the clear, sharp, central vision that is used to focus on objects that are in front of the eye. The rest of the retina provides side vision. AMD can create a blank or blind spot in the centre of vision, the side vision is not affected. The current medicine offers little in the way of effective therapies. In November 2004, Sirna Therapeutics launched the first clinical study of a chemically optimised siRNA in the treatment of AMD. Sirna-27 targets the vascular endothelial growth factor receptor-1 (VEGFR-1). The VEGFR–1 is located on vascular endothelial cells and is stimulated by VEGF and placental growth factor (PIGF), resulting in the growth of new blood vessels. Targeting the VEGF receptor results in down regulation of angiogenesis, initiated by VEGF and PIGF. Another approach is to silence the VEGF ligand. Acuity Pharmaceuticals used this strategy in the development of siRNA drugs in AMD treatment and envisiones to launch clinical trials in the near future.

GENETIC REPAIR MEDIATED BY RIBOZYMES

Diseases resulting from genetic disorders traditionally have been tried to therapy by addition of correct copies of abnormal genes to the genome. However, several hurdles associated with gene delivery or gene regulation still have to be overcome. To correct inherited diseases like Huntington's disease, Duchenne muscular dystrophy, hemophilia A or cystic fibrosis, huge genes need to be delivered to cells, what remains a big challenge. Furthermore, spatial regulation of gene expression is required. For example in cystic fibrosis, heterogenous expression profiles of cystic fibrosis transmembrane conductance regulator (CFTR) are required in various epithelia cells types to restore correct chloride conductance [178]. On the other hand, the safety of randomly transferred gene integration into patients DNA has to be considered [179].

In the past two decades, the understanding of the genetic basis of diseases as well as of the cellular processing of DNA and RNA has allowed for emergence of new techniques for genetic repair. Genetic repair instead of gene addition, nowadays seems to have great potential, since it conserves endogenous spatial and temporal gene regulation and simultaneously reduces the expression of the mutant gene.

Furthermore, transgene sizes are reduced rendering transfection more facile. Genetic repair can occur at gene as well as at transcript level, using DNA or RNA [180], or in a different context, a tRNA-splicing endonuclease [181] as repair agent. RNA-based technologies that modify genetic information at the level of genes or transcripts or that interfere with protein function are being developed [87]. In this review we concentrate on RNA-mediated gene repair techniques at the level of transcripts and genes. In that, genetic repair at the gene level is a rather controversial theme. It implicates permanent correction but targeting errors could be fatal. Therefore, transcript reprogramming seems to be safer.

RNA MEDIATED TRANSCRIPT REPAIR

Transcription in higher eukaryotes of most protein-coding genes produces a primary transcript (pre-mRNA) that is composed of introns, or non-coding regions, and exons, which are kept in the mature transcript or messenger RNA (Fig. (**10a**)). During a reaction termed *cis*-splicing, the spliceosome catalyses intron excision and ligation of the remaining exons (Fig. (**10b**)). RNA sequences like group I intron ribozymes found in lower eukaryotes catalyse their own excision out of the transcript in a related form of *cis*-splicing, recruiting a separate guanosine moiety as cofactor (Fig. (**11a**)). Furthermore, there is another, rather rare type of spli-

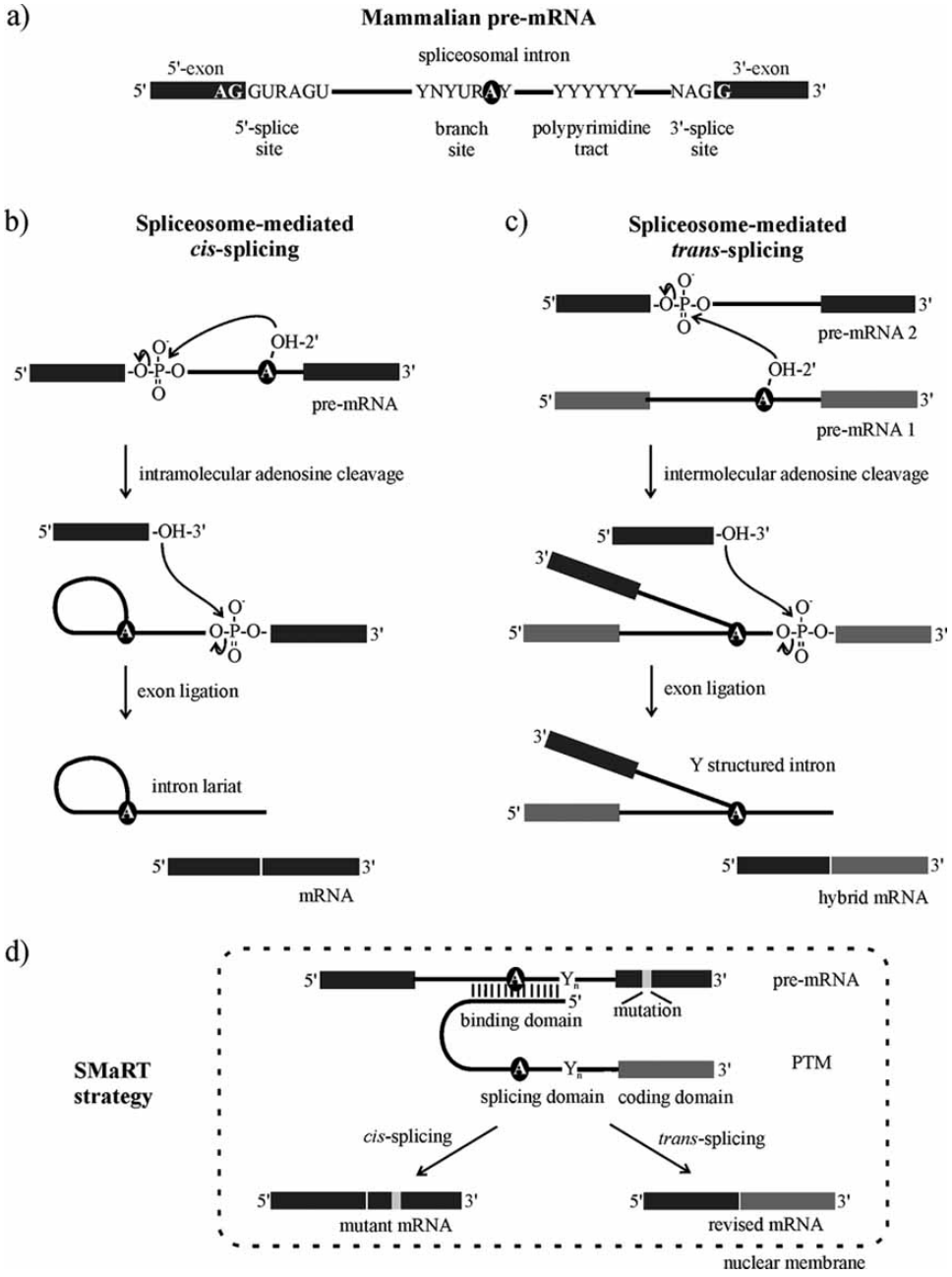

Fig. (10). Spliceosome-mediated splicing reactions. (a) Consensus sequence of the splice sites found in the major class of human introns (N = any nucleotide; Y = pyrimidine; R = purine) [183]. (b) Mechanism of cis-splicing. (c) Mechanism of trans-splicing. (d) SMaRT strategy: a pre-trans-splicing molecule (PTM) is used to mediate site-specific exon replacement within a target pre-mRNA using the cell own spliceosome machinery.

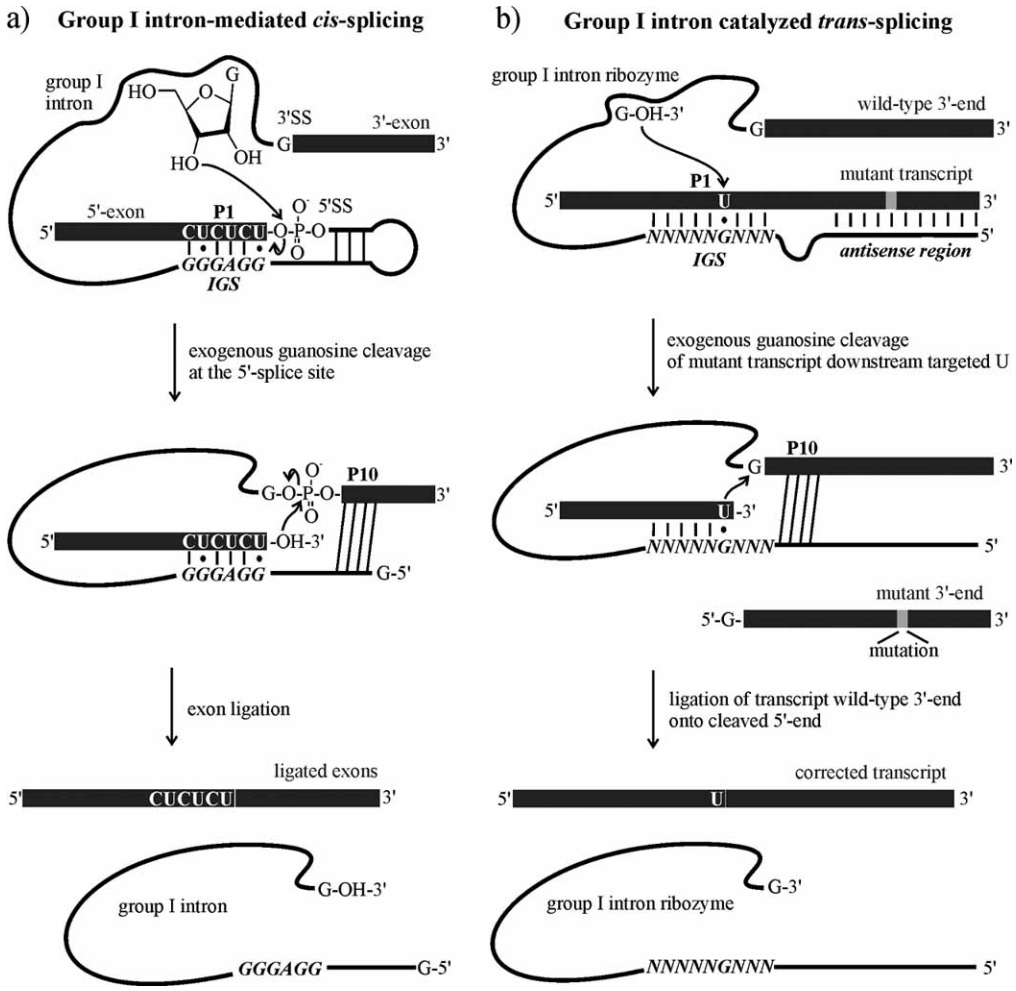

Fig. (11). Group I intron-mediated splicing reactions. a) Mechanism of *cis*-splicing. b) *Trans*-splicing reaction and ribozyme design for 3′-exon replacement. The 3′-end of a mutant transcript is replaced with a wild-type sequence carried by a modified group I intron ribozyme. The 5′SS in the mutant transcript is chosen at an uridine upstream of the mutation. In the ribozyme the 5′-exon is removed, the 3′-exon is replaced with the substrate wild-type 3′-end; the IGS is altered to allow formation of an elongated helix P1 (five base-pairs downstream and three base-pairs upstream of the G-U base-pair); four nucleotides upstream of the G-U base pair are modified to allow formation of helix P10; an antisense region of 5-200 nucleotides is added at the ribozyme 5′-end. The reaction mechanism is the same as for the cis-splicing reaction.

ceosome-mediated reaction called *trans*-splicing, occurring in lower as well as in higher eukaryotes. Here, two different pre-mRNAs are processed to produce a mature transcript containing exons from two different precursor molecules (Fig. (**10c**)).

A possibility to reprogram transcript sequences is to artificially induce a *trans*-splicing event, for example using the cells own spliceosome machinery to *trans*-splice new genetic information. Alternatively, a *trans*-splicing ribozyme carrying the transgene may be introduced into the cell. Both ways have been used to correct genetic information at the transcript level in therapeutic cell and animal models [182]. Alteration of pre-mRNA splicing can be also achieved by using RNA antisense molecules to block splicing sites as described above. Furthermore, some new strategies for RNA reprogramming are currently being developed.

Spliceosome-Mediated Trans-Splicing

In the nucleus of mammalian cells, *cis*-splicing is catalysed by the spliceosome, a huge enzymatic complex composed of many proteins and four small ribonucleoprotein particles [183]. The selection of the 5′- and 3′-splice site by the spliceosome is guided by conserved sequence elements which distinguish two intron classes: the major class (>99 %) called U2 dependent introns (Fig. (**10a**)), [183]) and a minor class called U12 dependent introns [184].

C*is*-splicing is performed through two transesterification reactions (Fig. (**10b**)). In the first step, the 2′-OH of a specific adenosine within the intron, called branch point, attacks the phosphate downstream of the 5′-exon, thus forming a branched structure and a free 5′-exon. In the second step, the 3′-OH group of the 5′-exon is ligated to the phosphate upstream of the 3′-exon and the intron is released as a lariat. *Trans*-splicing, in which an intron of a pre-mRNA interacts with an intron of another pre-mRNA to form a hybrid molecule (Fig. (**10c**)), was first described in lower eukaryotes [185,186]. Later, it was discovered also in higher eukaryotes, including rats and humans [187-189]. *Trans*-splicing remains a rarely detected event in mammalians, but a recent computational study suggests it to be implicated in the generation

of a larger number of mRNAs [190]. The reaction mechanism (Fig. (**10c**)) is analogous to the intramolecular reaction (Fig. (**10b**)): the 2′-OH function of the branch point adenosine of one molecule attacks the 5′-splice site of a second, and the free 5′-exon is ligated to the 3′-splice site of the first molecule. The released intron has a Y-structure instead of being a lariat. In 1999, Puttaraju *et al.* presented the first example of spliceosome-mediated RNA *trans*-splicing for therapeutic use (SMaRT™ strategy, Fig. (**10d**)), reprogramming the cancer-associated human chorionic gonadotropin β-polypeptide (CGB) mRNA [191]. Addition of an RNA effector or pre-*trans*-splicing molecule (PTM) to tumour cells resulted in the site-specific exon replacement in cell culture and in an animal model [191]. They further presented direct evidence for functional mRNA repair in human cells with the repair of a defective ß-galactosidase (*LacZ*) gene [192]. A PTM is constituted of three different domains (Fig. (**10d**)): the binding domain complementary to the target intron, the splicing domain containing splicing elements equivalent to those found in *cis*-splicing precursors (Fig. (**10a**)) and the coding domain carrying the correct exon to be *trans*-spliced to the target. The splicing domain usually has a 3′-splice site subdomain (with a branch point sequence, a polypyrimidine tract and a 3′-splice site), such that it can be *trans*-spliced to a 5′-splice site in the target. A PTM with a 5′-splice site domain has also been used *in vitro* to be *trans*-spliced to a 3′-splice site in the target pre-mRNA [193]. The typical size of the binding domain is 70-150 bases. To improve the level of target specificity, the target-binding domain has been elongated to 200 bases and more, whereas efficiency was maximal at 150 nucleotides [192]. To avoid unspecific splicing, an intra-molecular stem across the binding and the splicing domain can be engineered at the PTM 5′-end to mask the 3′-splice site from spliceosomal components until it binds its target [191,194]. The coding domain can be as large as 3.3 kb [195].

SMaRT has been applied to RNA-repair therapy for the treatment of genetic disorders [195-198] and suicide-gene therapy for the treatment of cancers [199,200] (Table **2**).

Efficiency remains low but phenotype correction is often higher [192,165,196]. Efficiency is dependent on many factors like concentration of the PTM and target, strength of PTM and target splice sites, accessibility of the binding domain or the kind of delivery. Target specificity is an even bigger problem. A negative assay has been reported [201]: Kikumori *et al.* designed an *in vitro* system to repair a mutant proto-oncogene sequence (RET: REarranged during Transfection). They noticed non-targeted *trans*-splicing into the RET exon and endogenously expressed mRNA, whereas specific *trans*-splicing was a rather rare event. Addition of a stem-loop structure in the PTM design failed to reduce non-specific splicing. Thus, it was concluded that overexpression of PTMs with a 3′-splice site results in promiscuous *trans*-splicing [201].

SMaRT is a promising strategy to reprogram RNA using the cells own machinery, which has been reviewed in more detail elsewhere [182,202-204]. However, there are still problems to be overcome. A limitation of the technique is for example the requirement of nuclear introns in the target, which exclude targeting of intronless genes and non-coding RNAs. Finally some more successful reports demonstrating target specificity are required to validate the strategy.

Group I Intron Ribozyme-Mediated Trans-Splicing

Group I intron ribozymes found in the ribosomal RNA of lower eukaryotes and some bacteria catalyse their own exci-

Table 2. Preclinical Relevant Applications of Spliceosome-Mediated RNA *Trans*-Splicing for Therapeutic Use (SMaRT™ strategy) for the Treatment of Genetic Disorders and Suicide-Gene Therapy for the Treatment of Cancers

Therapeutic model	Target transcript	Delivery	System	Functional repair	Ref.
Cystic Fibrosis (CF)	ΔF508 CFTR	AdV	human bronchial CF xenografts in nude mice	22 %*	[193]
		AAV	human CF polarized airway epithelia	14 %*	[196]
Hemophilia A	Coagulation factor VIII	naked DNA	F8 knockout mice	13 %*	[194]
		AdV		11 %*	
X-linked Immuno-deficiency	CD40 ligand (CD40L)	lentiviral vector, *ex vivo*	CD40L knockout mice	antigen specific response	[195]
Cancer	A-Cobratoxin**	naked DNA	in mice	mice death	[197]
	Shigatoxin 1A1**	AdV	human tumor in nude mice	inhibition of tumor growth	[198]

* Maximal percentage of correction over time related to normal protein level.

** segmental *trans*-splicing: 5′- and 3′-exon are both inserted in the cell, thus reconstituting the toxin transcript *in situ*.

AdV: recombinant adenovirus

AAV: recombinant adeno-associated virus

sion (*cis*-splicing) through two transesterification reactions (Fig. (**11a**)) [205].

In the first splicing step, the 5′-splice site downstream of a G-U wobble base pair in helix P1 (formed by base-pairing between the 5′-exon and the internal guide sequence (IGS) of the intron) is cleaved by an exogenous guanosine lofted within the intron. After cleavage, helix P1 is displaced to allow formation of the P10 helix between the IGS and four nucleotides downstream of the 3′-splice site. In the second splicing step, the 3′-OH of the terminal uridine of the 5′-exon is ligated to the phosphate upstream of the 3′-exon. A truncated form of the *cis*-splicing intron from *Tetrahymena thermophila* without 5′-exon was shown to *trans*-splice a sequence attached to the ribozyme 3′-end, to a target mimicking the 5′-exon [43]. The ribozyme IGS may be modified to base pair with a target mRNA, as long as a G-U base pair is maintained at the 5′-splice site [43]. Ribozyme-mediated RNA repair by 3′-exon replacement was demonstrated in *Escherichia coli* [43] and in cultured mammalian cells [206] with the repair of truncated *lacZ* transcripts. Following co-transfection of ribozyme and substrate containing plasmids in mammalian cells, the ribozyme revised up to 49 % of the truncated *lacZ* mRNAs [207]. Following this initial study, ribozymes with improved biological activity have been engineered (Fig. (**11b**)) [208]. As an essential implement of *trans*-splicing ribozyme design, an uridine within the target has to be chosen upstream of the mutation thus defining the 5′-splice site. This is the sole sequence requirement in the target RNA. Ribozyme libraries have been used to map accessible uridines *in vitro* [209-211] and *in vivo* [212]. In the ribozyme, the IGS must be altered to allow formation of helix P1 (Fig. (**11b**)): a G-U base-pair at the intended 5′-splice site, five base-pairs downstream and three base-pairs upstream of this G-U base-pair [208]. The ribozyme sequence must also be altered upstream of the G-U base pair to allow formation of the four base-pair helix P10 [208]. Finally, the inclusion of an antisense region of 5-200 nucleotides at the 5′-end of the ribozyme improve both efficiency and specificity for the intended target [208,213]. Alternatively, a group I intron ribozyme catalysing 5′-exon replacement *in vitro* has recently been reported [214]. *Trans*-splicing ribozymes have been successfully employed to revise mutant transcripts (Table **3**) associated with human genetic diseases in mammalian cells [209,211,213,215,216] or cancer associated transcripts [210,217,218]. Other approaches use *trans*-splicing to replace viral transcripts or oncogenes with sequences that induce cell death [219-222]. For details see reviews [65,223].

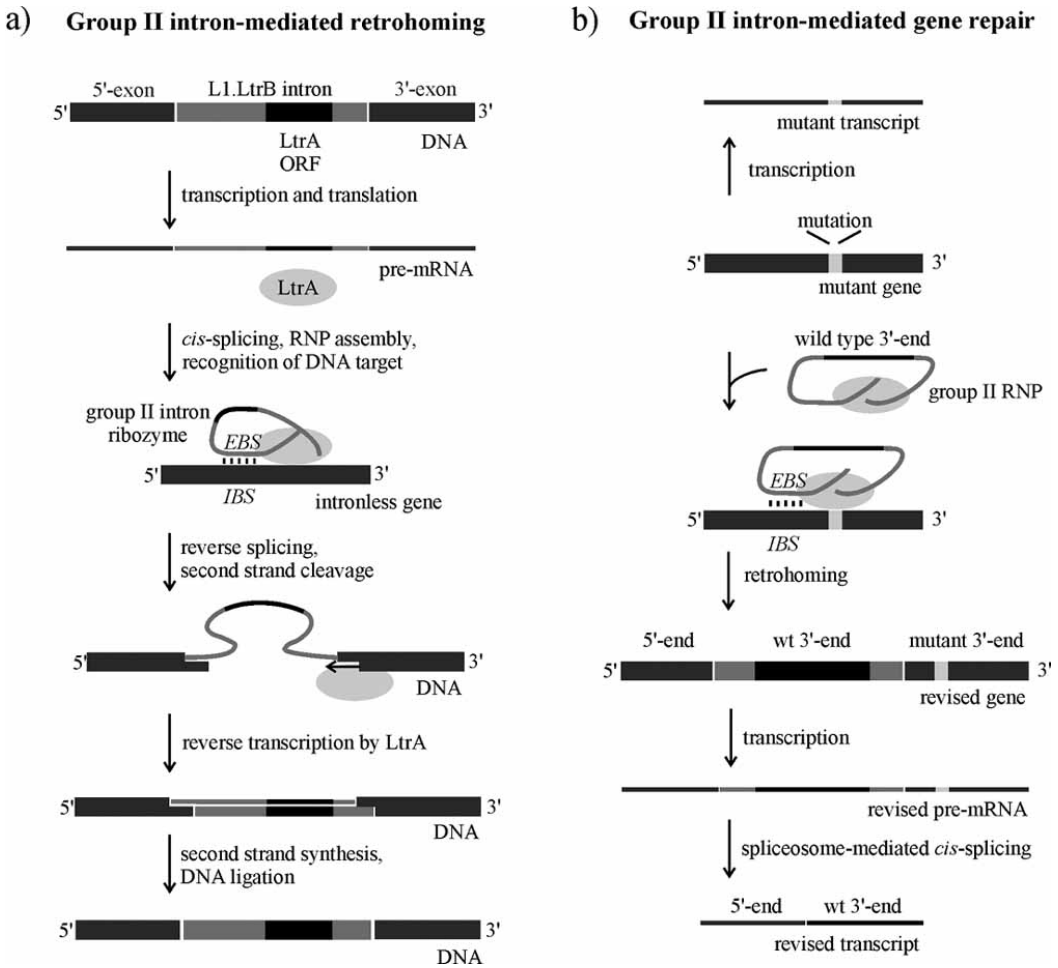

Fig. (12). Group II intron retrohoming reaction. a) Insertion pathway of the group II intron ribozyme of the *Lactococcus lactis* retrotransposon *Ll.LtrB* into a specific intron less gene. [223]. b) Group II intron-mediated gene repair. An IBS is chosen in the DNA target upstream of the mutation [223]. ORF: open reading frame; EBS: exon-binding site; IBS: intron-binding site.

Table 3. Therapeutic Relevant Applications of Group I Intron Ribozymes for the Treatment of Inherited Genetic Disorders, Viral Infections and Cancers. All Ribozymes were Transfected to Cells Over Direct Plasmid Injection

Therapeutic model	Target transcript	Strategy	System	Results	Ref.
Myotonic Dystrophy	myotonic dystrophy protein kinase (DMPK)	replacement with shorter trinucleotide repeat (TNR)	mammalian cells	transcripts shortened from12 to 5 TNR	[213]
Sickle Cell Anemia	sickle β-globin	replacement with γ-globin	erythrocyte precursors isolated from patients	detection of repair product	[207, 209]
			HEK293 cells	10 % RNA repair	[211]
Myotonia Congenita	mutant canine skeletal chloride channel	replacement with wt	HEK293 cells	restoration of function in 18 % of cells	[214]
Hepatitis C	hepatitis C virus internal ribosome entry site (IRES)	replacement with diphtheria toxin A chain	HEK293 cells	induction of apoptosis	[217, 218]
Cancer	mutant p53	replacement with wt	human cancer cells	induction of apoptosis	[208, 215]]
	mutant p16	replacement with wt	pancreatic cancer cells	restoration of protein synthesis	[216]
	tumor specific genes	replacement with cytotoxic gene	human cancer cells	induction of apoptosis	[219, 220]

Other Strategies of Ribozyme-Mediated Revision of RNA

Ribozymes have been engineered to repair RNA through removal or addition of small internal fragments as well as single nucleotides based on the group I intron ribozyme [224-226] or on the hairpin ribozyme [227]. These systems are true repair systems but until now have been applied only *in vitro*.

All methods of transcript correction *in vivo* have in common, that, the effector molecule has to be stably expressed in host cells. Therapy efficiency is therefore strongly dependent on effector molecule delivery [reviewed in 65]. For example, persistent expression can be achieved for up to 4.5 kb effector molecules, when delivered with adeno-associated viruses. One way to enhance repair efficiency is to co-localise the effector molecule with the substrate at both cellular (tissue) and sub-cellular (cytoplasm versus nucleus) level [228]. Furthermore, the use of two group I ribozymes targeting different sites on the same transcript was also shown to enhance the overall repair efficiency [211]. Another factor affecting repair efficiency is the type of expression cassette promotor used [reviewed in 65].

RNA-MEDIATED GENE REPAIR

Site-specific DNA repair implicates long term efficiency and maintenance of cell regulation and is therefore a big challenge. In 1996, Cole Strauss *et al.* have shown that small RNA-DNA chimera oligonucleotides can induce homologous recombination pathways in mammalian cells and thus are able to repair gene point mutations [229]. This result had a big impact on the gene therapy community, but a few years later the method appeared not to be generally applicable [230]. Nowadays, DNA-based methods are preferably used to induce homologous recombination pathways [231]. Another emerging method is based on ribozyme-mediated DNA insertion or retrohoming [223].

Retrohoming Group II Introns

Group II introns are *cis*-splicing ribozymes, which follow the same reaction mechanism as spliceosome-mediated *cis*-splicing (Fig. (**10b**)) [223]. The Group II intron ribozyme of the long terminal repeat retrotransposon *Ll.LtrB* of *Lactococcus lactis* is a mobile RNA genetic element that inserts into a specific intron less gene through a process called retrohoming (Fig. (**12a**)) [232].

After transcription of the LtrB precursor RNA, an intron-encoded protein called LtrA is translated. The multifunctional protein facilitates *cis*-splicing of the catalytic intron and then forms with the excised intron a ribonucleoprotein complex having endonuclease and integrase activity [233]. Target recognition is effectuated by the intron *via* base pairing between the exon-binding site (EBS) in the intron and the intron-binding site (IBS) in the intronless gene as well as by the LtrA protein that interacts with the target at nucleotides flanking the EBS-IBS pairing [234]. After intron reverse-splicing into the DNA, LtrA cleaves the DNA strand opposite the insertion site, nine nucleotides downstream of the intron and reverse transcribes the intron. The cellular machinery completes second-strand synthesis and ligation of the DNA [235]. Ribozymes may be retargeted to new target sites in DNA by altering the sequence of the EBS [4,223, 234]. Retrohoming introns have been redirected to disrupt pathogenic genes with efficiency in bacteria as high as 60 % as well as in human cells [4]. Recently, Sullenger and coworkers succeeded in repairing mutant *lacZ* and *β-globin* genes with high fidelity in model bacteria systems [236]. They replaced the LtrA sequence of the *L. lactis* group II

intron with the wild-type *lacZ* exon sequence. The LtrA sequence was delivered to the cell as the intron encoded plasmid. The ribozyme sequence was modified to integrate into the mutant target gene and to deliver the wild-type sequence (Fig. (**12b**)). Upon co-transfection of target and intron plasmids in *E. coli,* 60% *lacZ* gene repair was achieved with high specificity. The authors applied the same technique with the *β-globin* gene but obtained only up to 2 % gene repair in *E. coli*, whereby the repaired genes were not in all cases translatable in mammalian cells [236].

Because genes are modified, transient expression of the ribonucleoprotein complex should be sufficient to permanently alter a cell population. Retropositioning of genetic elements in repairing defect genes at the DNA level unifies advantages of both, conventional gene therapy and RNA repair. It is an elegant method, which however, is still at the beginning of development.

In summary, RNA-based technologies to reprogram transcripts and genes for treatment of human diseases are just emerging. There is a great interest, since these strategies allow for maintaining of the cells own spatial and temporal transcript regulation. However, none of these methods would offer a universal therapeutic molecule targeting a specific disorder like in conventional gene therapy. A therapeutic molecule pool bank addressed to the diversity of patient mutations would be required for the methods to be universal. Several scientific and technical challenges must first be addressed and surmounted before thinking of clinical trials. Low repair efficiencies may not be a major problem, since many genetic disorders may be corrected by the restoration of even low levels of normal protein or enzyme activities. In the case of sickle cell anaemia, expression of γ-globin at a level of about 10-20 % that of sickle γ-globin is sufficient to overcome the disease symptoms [237]. Similarly, in cystic fibrosis, expression of functional CFTR in approximately 8-15 % of epithelial cells has been shown to restore normal chloride secretion [178]. Nevertheless, target specificity is still a major hurdle and delivery systems allowing persistent repair have to be tested.

ABBREVIATIONS

2′-MOE = 2′-O-methoxyethyl

2′-OMe = 2′-O-methyl

ADA = Adenosine deaminase

Ago = Argonaute

AMD = Age related macular degeneration

CFTR = Cystic fibrosis transmembrane conductance regulator

CML = Chronic myelogenous leukemia

DMD = Duchenne muscular dystrophy

E. coli = Escherichia coli

EBS = Exon-binding site

EBV = Epstein-Barr virus

FANA = 2′-deoxy-2′-fluoro-β-D-arabinonucleotide

HBcAg = Hepatitis B core antigen

HBsAg = Hepatitis B surface antigen

HBV = Hepatitis B virus

HCV = Hepatitis C virus

HD = Hepatitis delta ribozyme

HH = Hammerhead ribozyme

HIV = Human immunodificiency virus

hnRNP = Heterogeneous nuclear ribonucleoprotein

HP = Hairpin ribozyme

IBS = Intron-binding site

IGS = Internal guide sequence

IL = Interleucin

kb = Kilo base

L. lactis = Lactococcus lactis

miRNA = micro RNA

NPC = Nasophyngeal carcinoma

nt = Nucleotide(s)

ORF = Open reading frame

PACT = protein activator of PKR

PAZ = Piwi Argonaute Zwille

PIGF = Placental growth factor

PKR = Protein kinase R

PTB-1B = Protein tyrosine phosphatase 1B

PTEN = Phosphatase and tensin homologue

PTM = Pre-trans-splicing molecule

rAsONs = Respirable antisense oligonucleotides

RET = Rearranged during transfection

RISC = RNA induced silencing complex

RITS = RNA-induced initiation of translational gene silencing

RNAi = RNA interference

RNP = Ribonucleoprotein particle

siRNA = Short interfering RNA

SMaRT = Spliceosome-mediated RNA trans-splicing

SS = Splice site

TFO = Triplex forming oligonucleotide

TRBP = TAR RNA-binding protein

VEGF = Vascular endothelial growth factor

VEGFR = Vascular endothelial growth factor receptor

VS = Varkud satellite ribozyme

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