RNA-Mediated Therapeutics: From Gene Inactivation to Clinical Application

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Abstract: The specific targeting and inactivation of gene expression represents nowdays the goal of the mainstream basic and applied biomedical research. Both researchers and pharmaceutical companies, taking advantage of the vast amount of genomic data, have been focusing on effective endogenous mechanisms of the cell that can be used against abnormal gene expression. In this context, RNA represents a key molecule that serves both as tool and target for deploying molecular strategies based on the suppression of genes of interest. The main RNA-mediated therapeutic methodologies, deriving from studies on catalytic activity of ribozymes, blockage of mRNA translation and the recently identified RNA interference, will be discussed in an effort to understand the utilities of RNA as a central molecule during gene expression.

Keywords: Ribozymes, hammerhead, hairpin, self splicing introns, RNA interference, antisense, RNase P, M1 RNA, gene therapy, gene inactivation, clinical trials, HIV, cancer.

I. INTRODUCTION

In the post-genomic era the specific and rational targeting of essential genes has become the driving force for the scientists and the pharmaceutical companies. Most of the accumulated bioinformatic data on the published genomes and especially on the human genome has been focused on unraveling molecular targets that could be easily blocked either in the genomic or the expression level. However, until recently, the tools for such specific gene inactivation were limited. The discovery of catalytic RNA, some 25 years ago, gave much hope on the deployment of new tools based on a nucleic acid level and not on a peptide level, for therapy. The ability of small RNA molecules to cleave, thus inactivating, specific ribonucleic targets, revitalized the scientific idea of specific gene inactivation and eventually gene therapy. Few years before that, the first demonstration of a small antisense oligonucleotide that could specifically block Rous sarcoma virus mRNA had been published, giving rise to the important new field of antisense technology [1].

The most recent discovery of RNA interference (RNAi) became an additional valuable tool in the researchers' toolbox. It is nowadays, routinely used in many aspects of biological research, from the developmental biology to infection and cancer, in an effort to throw light on basic biological processes in a more specific way [2].

Although contemporary drug design and discovery is driven by the power that scientists have to easily identify and evaluate a molecular target, the major challenge that still remains is basically the correct interpretation of the genetic information. Currently, targeting mRNA for both target validation and as a therapeutic strategy is being pursued using ribozymes, antisense approaches and the recently identified RNA interference (RNAi). In the context of specific gene inactivation as a therapeutic method and eventually as a specific drug agent, RNA moved once more in the spotlight [3]. However the road from bench discoveries to clinical trials and successful delivery of RNA as novel, specific and efficient therapeutic agent still seems very long. In this article we will summarize the attempts for RNA-based therapies, namely small ribozymes, RNase P, group I and II introns, RNAi and antisense technologies.

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II. SMALL RIBOZYMES

The class of small ribozymes comprises a few well characterized catalytic RNA molecules such as hammerhead, hairpin, *Hepatitis* delta virus (HDV), and *Neurospora* varkud satellite (VS). All naturally occurring small ribozymes undergo a site-specific self-cleavage in the presence of magnesium ions. The self-cleavage is a transesterification reaction using an in-line S_N2 mechanism, where the scissile phosphorus bond is attacked by the 2'-hydroxyl group belonging to the 5' ribose. The cleavage yields 2'-3'-cyclic phosphate and 5'-hydroxyl termini. Hammerhead and hairpin ribozymes have found extensive use in gene silencing applications such as cleavage of viral RNAs, downregulation of oncogenic mRNAs, or the control of gene expression *in vivo*, and will be the main focus points of this section.

Hammerhead Ribozyme

The hammerhead ribozyme is the smallest naturally occurring catalytic RNA. It was initially found as a catalytic motif in several plant pathogen RNAs, such as the the plus strand of satellite RNA of Tobacco ring spot virus (+sTRSV) and other plant viruses [4-6], and genomic RNA of plant viroids [7-9]. Additionally, active hammerhead domains have been characterized in RNA transcripts of animal satellite DNA [10-12]. All hammerhead motifs are involved in the maturation process of long multimeric transcripts. The replication of the plant pathogens' genomes occurs by a

1568-0266/06 $50.00+.00

rolling cycle mechanism [13]. Their circular RNA genome is repeatedly copied into multimeric transcripts that are converted into genome-length strands. It has been shown that this step involves in several cases a self-cleavage reaction catalyzed by a hammerhead ribozyme [14].

Hammerhead ribozyme requires divalent metal ions for structural and functional purposes, although it was shown that higher concentrations of some monovalent ions (Li^+, NH_4^+) can substitute for Mg^{2+} [15].

The secondary structure is common in all hammerhead motifs, comprising three helical stems, numbered I to III, connected by two single stranded regions which harbour the catalytic core [16] (Fig. **1**). Helices consist of non-conserved nucleotides, while the single stranded regions contain several invariant nucleotides important for catalysis. Natural hammerhead ribozymes can be transformed into true multiple-turnover catalysts capable of targeting and cleaving *in trans* various RNA molecules (substrates) such as pathogenic mRNAs and the genomic RNA of retroviruses [17,18].

In most *trans*-cleaving hammerhead ribozymes helix II is formed intramolecularly, while helices I and III are formed by the intermolecular interaction between the catalyst (binding arms) and the complementary sequence on the substrate. The lack of conserved nucleotides in stems I and III allows the design of ribozymes specific for any desired target sequence. The most commonly used hammerhead motif has a length of approximately 35 nucleotides depending on the length of the substrate binding arms (Fig. (**1**)). Stems I and III flank the cleavage site on the target molecule. Mutagenesis studies have revealed that the consensus sequence 5' upstream of the cleavage site is NUH (N=any nucleotide and H=A, C or U) [19], although there are data supporting a reformulation of this canon to NHH [20].

Hairpin Ribozyme

The hairpin motif was firstly identified in the minus strand of the satellite RNA of tobacco ring spot virus (-sTRSV) the same RNA genome that harbors a hammerhead ribozyme in its positive strand [21,22]. Hairpin ribozyme is responsible for the cleavage of multimeric genomic products as described for hammerhead, but it also ligates the processed monomers, generating circular RNA molecules [for review see 23]. The ribozyme has a functional length of 50 nucleotides, and can cleave specifically a target RNA sequence which recognizes by base-pairing. The catalytic motif consists of two independently folding domains (I, II) connected in its natural form by a four-way junction which can be reduced to a hinge in a minimal *trans*-cleaving form. Domain I is formed by the ribozyme and the base-paired substrate and contains two helices (1 and 2) separated by an internal loop (loop A). Domain II is composed solely by the ribozyme and has a similar configuration (helices 3, 4 and loop B) (Fig. (**2**)).

Loops A and B contain conserved nucleotides essential for activity. Extensive non-canonical base-pairing is observed within those loops. To achieve catalytic activity the ribozyme adopts a compact conformation ("docked state"), which facilitates Watson-Crick interaction between the two loops [24]. Mutagenesis studies has established that an N*GUC sequence at the target cleavage site (the asterisk represents the scissile bond) is more efficiently cleaved *in vivo* [25] and this is followed as a rule for the design of a hairpin *trans*-acting ribozymes [26]. The reaction is metal ion independent, but Mg^{2+} contributes to structure stabilization [27-28].

Designing and Testing Ribozymes - Requirements and Considerations

Ribozymes based on hammerhead and hairpin catalytic motifs are valuable tools to a wide range of gene therapy applications. Ribozymes display significant advantages over other approaches: a) they are small in size and possess a simple catalytic domain which makes them more versatile; b) they are capable of directly cleaving the target themselves and do not depend on the activation of other enzymes; c) they bind and cleave RNA targets with high specificity, e.g. they can discriminate mutant RNA sequences from wild type; d) they can be tailored to target viral RNAs as well as cellular mRNAs; e) they have high turnover rate; f) they are

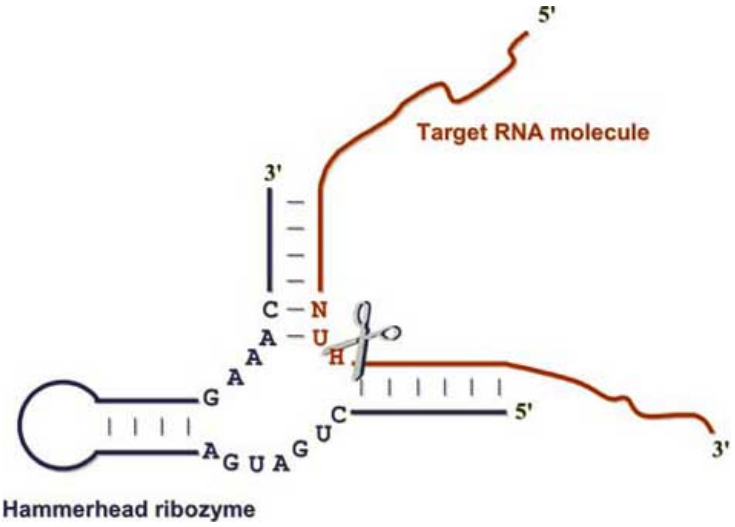

Fig. (1). Secondary structure of a trans-cleaving hammerhead ribozyme. The ribozyme and the RNA target sequences are depicted in dark and light grey respectively. The scissors indicate the cleavage site.

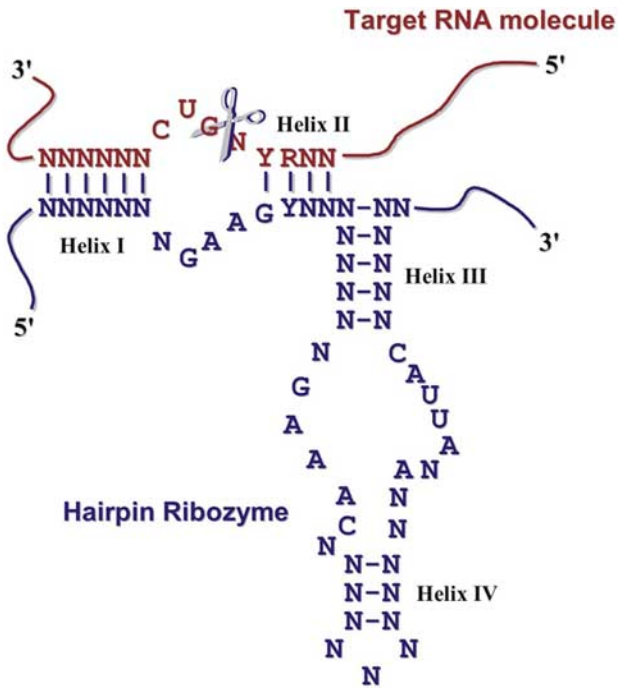

Fig. (2). Secondary structure of a *trans*-cleaving hairpin ribozyme.

effective at low concentrations; g) they can be allosterically controlled; h) they can be introduced into cells either exogenously as a classical drug or endogenously as a transfected gene.

These ribozymes have also certain disadvantages and limitations that have to be taken into consideration: a) the target must contain a specific sequence that is recognized as the cleavage site; b) RNA molecules are very sensitive to abundant RNase activity that can affect their *in vivo* half life; c) they can bind proteins non-specifically leading to reduced effectiveness; d) they need to be co-localized with their target inside cells.

In the following section we will describe how the aforementioned particular characteristics are employed in the design and testing of a candidate ribozyme.

Target site Selection

As it was described earlier, hairpin and hammerhead ribozymes require for cleavage a specific triplet on the substrate RNA molecule, 5'-N*GUC and 5'-NUH*N respectively. Therefore, the procedure begins with the identification of cleavage sites, which is usually performed *in silico* [29]. It is important that a candidate site must not be obstructed by target RNA secondary structure. The accessibility of the target site can be initially assessed by secondary structure analysis algorithms [29], chemical modification [30] and/or RNase H mapping [31]. Additionally, the flanking regions must provide adequate annealing energies and sequence uniqueness. These two features ensure that the binding of the ribozyme onto the target sequence is specific, fast and strong. The strength of the binding must be high enough so that the ribozyme does not dissociates prematurely, and on the other hand, low enough to be released rapidly after cleavage, in order to process another substrate (high turnover rate) [32,33].

Testing Ribozymes In Vitro

Once the site and flanking regions are determined, the resulting ribozyme must be synthesized and tested *in vitro*, under standard conditions, for the efficient cleavage of the target RNA. Nevertheless, efficient *in vitro* activity does not ensure satisfactory *in vivo* performance, and, surpisingly, in some cases ribozymes that exhibit low *in vitro* activity show high *in vivo* efficiency.

Testing Ribozymes In Vivo

A cell culture system is the next testing ground for a candidate therapeutic ribozyme. For *in vivo* application, the ribozyme can be administered directly as a classical drug, or it can be introduced into cells as a recombinant gene on an appropriate vector.

In the first case, the ribozyme is usually delivered into cells by cationic lipids or liposomes [34]. Additionally, the chemically synthesized ribozyme must be protected from degradation by ubiquitous nucleases. This is achieved by chemical modification of the ribozyme, such as thio-modification, methylation or alkylation at the 2´ position of the ribose ring. Such chemically modified ribozymes are being evaluated in clinical trials [35]. However, it has been observed that such modifications result in significant cellular toxicity [36] and non-specific binding to proteins. Among the vehicles used for ribozyme delivery, poly(ethylene imine) when coupled to ribozymes stabilizes them against degradation by nucleases and facilitates efficient cellular uptake from endosomal compartments, thus providing a novel method for exogenous delivery of ribozymes without chemical modification [37].

In the second case the ribozyme gene is introduced into cells by vector-based methods, and is transcribed by the host cell machinery. Several viral vectors, developed and used for gene therapy, have been used also for ribozyme gene delivery, including adenovirus, retrovirus, adeno-associated virus, and lentivirus [38-42]. Unlike murine retroviral vectors, lentiviruses offer greater therapeutic potential because they can achieve effective and sustained transduction and expression of therapeutic genes in nondividing cells [42]. In addition, due to safety considerations concerning the use of viral vectors, the design and use of artificial non-viral vectors have recently gained ground [43,44]. For the *in vivo* transcription to be achieved, the gene must be downstream of a promoter able to produce large (therapeutic) amounts of the ribozyme. The promoters of RNA polymerases II and III have been used for this purpose, with the latter being the current choice as it promotes the transcription of small RNA genes in levels 1 to 3 orders of magnitude higher than that of polymerase II [45, 46]. In an effort to improve ribozyme stability and colocalization with the RNA target molecule, the ribozyme genes have been fused to tRNA, U1 and U6 snRNA genes and expressed *in vivo* as chimeric molecules with significant results [47]. In addition, a novel and promising approach is the incorporation of a helicase recruiting domain to the ribozyme molecule [48]. This ribozyme-helicase complex is much more efficient than conventional

ribozymes in cleaving not only accessible but secondary structure obstructed sites as well.

Therapeutical Applications of *Trans*-Acting Ribozymes Based on Hammerhead and Hairpin Motifs

The aforementioned designing and testing strategies have been implemented in the use of hammerhead and hairpin ribozymes as tools for specific gene inactivation in the fields of cancer, viral infections, and other diseases caused by various genetic disorders.

Antiviral Applications

1. Human Immunodeficiency Virus (HIV)

Approximately one third of the total publications on ribozyme-mediated gene therapy concern the inhibition of human immunodeficiency virus (mostly HIV-1) infection and replication. The primary targets for anti-HIV ribozymes are usually conserved elements critical for the virus life cycle, and comprise the long terminal repeat (LTR) (which includes the 5' leader sequence), the packaging sequence (ψ), and the mRNAs of *tat*, *rev*, *env*, *gag* and *pol* genes [49]. Most of the clinical approaches utilize *ex vivo* transduction of stem cells with retroviral or adeno-associated viral vectors that are later on infused into patients [50]. The rationale for this is that transduced stem cells will be repositioned in the bone marrow compartment, with subsequent proliferation and differentiation to give rise to a variety of hematopoietic lineages including $CD4^+$ T-lymphocytes and macrophages.

The highly conserved LTR region acts as a promoter of viral transcription, and has been the target of many hammerhead and hairpin implementations with good results [51-55]. The 5' leader sequence of HIV-1 is a desirable target due to its presence in all HIV-1 RNA transcripts. Its cleavage inhibits expression of both early and late viral gene products [53]. T-cell lines that express U5 targeted ribozymes are protected from HIV infection (preintegration effect) [54,55]. Moreover, HeLa $CD4^+$ and T cells expressing an LTR targeted hammerhead ribozyme that can be localized into the nucleolus, exhibit dramatically suppressed HIV-1 replication [56].

The (ψ) site is a stretch of approximately 120 nucleotides almost absolutely conserved among 18 HIV-1 strains [57], essential for RNA packaging during virus assembly. This site was the target for a hammerhead derived ribozyme that was able to inhibit HIV-1 infectivity and replication in the human T-cell line SupT1 [58].

Tat gene regulates the transcription of viral DNA into RNA. Endogenous expression of an anti-*tat* ribozyme can substantially inhibit HIV replication [59-62]. A hammerhead based anti-*tat* ribozyme carried on retroviral vector LNL6 named RRz2 has shown effectiveness in a range of test systems [60-62]. More specifically, Wang and colleagues observed increased cell viability in the ribozyme-transduced HIV-1-infected peripheral blood lymphocytes (PBLs), and marked inhibition of viral replication in T cell lines [62]. Subsequently, RRz2 has progressed from laboratory to clinical evaluation (phase I studies). In two long-term phase I clinical trials (that lasted approximately 4 years each), RRz2 was transduced into $CD4^+$ PBL and $CD34^+$ haematopoietic progenitor cells (HPC), which were afterwards infused into HIV-1-infected patients. The studies have shown that the ribozyme was stably expressed and that the gene transfer procedure was safe, and technically feasible. Moreover, no target site mutations that could confer resistance to the drug have occurred [63,64]. These results have provided the basis for designing and implementing a phase II clinical trial to further evaluate RRz2 as an anti-HIV tool.

Rev protein binds to RRE (*rev* responsive element) present in the spliced and unspliced viral RNA molecules and facilitates their transport through the nucleus membrane into the cytoplasm where they are translated [65]. *Rev* mRNA and RRE have been also selected as ribozyme targets, demonstrating the usefulness of this approach for inhibition of HIV replication [66-70]. Common regions of partially overlapping *Tat* and *rev* mRNAs have also been used as targets for ribozyme action [67-69].

The *env* gene encodes the gp160 polypeptide precursor containing the exterior gp120 and the transmembrane gp41 proteins. These are crucial for the attachment and entry of the HIV virions into $CD4^+$ T cells [65]. Conserved sites on the *env* coding region have been targeted by hammerhead ribozymes [71-72]. Multimeric hammerhead ribozymes targeting nine highly conserved sites within the HIV-1 envelope (Env) coding region proved more efficient than single site targeted monomeric ones in inhibiting HIV replication *in vivo* [72].

Important players in the mechanism underlying virion attachment and entry are the $CD4^+$ cellular receptors. CD4 and transmembrane chemokine receptors CCR5 and CXCR4 are the main HIV-1 receptors *in vivo* [65]. Cellular mRNAs appear more advantageous as gene therapy targets, because they exhibit significantly lower mutation rates than viral genes [73]. Moreover, while CCR5 appears to be more an attractive target than CXCR4 for anti-HIV-1 therapeutics [74], both CCR5 and CXCR4 are recently being used for this purpose with very promising results [75-80].

In general, it is expected that combinations of ribozymes targeting various genes of the HIV-1 replicative cycle are more likely to be effective than monomeric, single site targeted ribozymes. Additionally, the use of these tools would be best employed in conjunction with other therapies (RNA aptamers - decoys, RNAi methods, classic anti-HIV drugs) having a final synergistic effect. While such strategies may not completely eliminate HIV infection, such combination therapies may greatly increase $CD4^+$ lymphocyte survival.

2. Hepatitis C Virus (HCV)

Chronic infection by HCV can cause serious health problems including cirrhosis and hepatocellular carcinoma. HCV is an RNA virus, and has been one of the main subjects of ribozyme mediated gene therapy [81]. This virus is highly prone to mutation and poses a challenge to any therapeutical approach due to emergence of escape mutants. However, 5' and 3' untranslated regions (UTRs) of the virus genome display high degree of conservation among all HCV genotypes [82], and thus are suitable for ribozyme target sites [83]. 5' UTR contains an internal ribosome entry site (IRES) that plays a pivotal role for the translation of the viral genes. It has been shown in several studies that the 5' and 3'

UTR regions can be successfully targeted by hammerhead and hairpin originated ribozymes resulting in reduction of virus genes expression and inhibition of virus replication [84-87].

HEPTAZYME is a hammerhead derived, chemically modified ribozyme developed from Ribozyme Pharmaceuticals Inc. (presently Sirna Therapeutics). This ribozyme is exogenously delivered and targets the 5' UTR of HCV genome. HEPTAZYME was evaluated in phase I and phase II clinical studies alone and in combination with type I interferon [88]. Despite encouraging results showing moderate reduction of HCV RNA levels in patients' sera, the company decided to discontinue further development of the drug.

3. Hepatitis B Virus (HBV)

HBV infection is a major health problem worldwide, and is frequently associated with cirrhosis and hepatocellular carcinoma. HBV replicates its genome from pre-genomic RNA, thus presenting a suitable ribozyme target. The viral genome encodes four proteins: surface protein (S), core protein (C), polymerase (P) and X protein (HBx). HBx is a transcriptional activator protein, which stimulates not only all the HBV promoters, but also a wide range of other viral and cellular promoters. It is involved in virus replication and pathogenicity. HBx mRNA has served as a ribozyme target due to the gene's conservation and to the protein's central role in the HBV associated disease. Various anti-HBx hammerhead and hairpin implementations have successfully reduced the levels of viral particle production *in vivo* [89,90]. Other sites located on HBV S gene [90], pre-genomic mRNA [91,92], C gene [89,93], and polymerase gene [94] have been targeted as well, with interesting results.

4. Other Viruses

Hairpin and hammerhead cleavage has also been used in order to cope with infections by other viruses such as influenza virus [95], lymphocytic choriomeningitis virus (LCMV) [96], mumps virus [97], alphavirus [98], and bovine leukaemia virus [99].

Small Ribozymes as Tools Against Neoplastic Pathologies

The ability of ribozymes to target specific nucleic acid sequences could not be overlooked by researchers fighting cancer worldwide. Many hammerhead motifs have been used to target overexpressed or mutated oncogenes in an effort to suppress the malignant phenotype. A few well studied examples are presented below.

Mutations of the *ras* oncogene family are the most common alteration found in human cancers. *Ras* oncogene shows a high frequency of single base mutations, usually residing at codon 12 that cause tumorigenicity by sustaining unregulated signalling to downstream effectors. Such mutations are ideal targets of ribozyme-mediated interventions, because ribozymes have the ability to discriminate the mutated from the wild type mRNA, and subsequently cleave the former. This attribute was tested by targeting point mutations of K-*ras* [100-102], H-*ras* [103,104], and N-*ras* [105] in a series of recent publications, and results show reversal of the malignant phenotype.

Mutated variants of the tyrosine kinase receptors of the ErbB (HER) family frequently coincide with an aggressive clinical course of certain human adenocarcinomas. Targeting ErbB2 (HER-2) with endogenous expressed hammerhead ribozymes in ovarian [106,107], breast [108], bladder [109], gastric [110], and pancreatic cancer [111] has effectively inhibited cancerous cell proliferation and decreased tumour growth *in vivo*.

Human Papillomavirus (HPV) is related to more than 90% of cervical cancer. The virus is shown to be essential for the induction and maintenance of the malignant phenotype. Central role to this phenomenon play two early expressed viral genes, E6 and E7. Inhibition of E6/E7 expression by ribozyme was shown feasible and resulted in the reversal of the transformed phenotype, probably due to elevated expression of p53, Rb, c-myc and bcl-2 that lead cancer cells to apoptosis [112-115].

Angiogenesis is a requisite for the sustaining of a growing tumour. This makes vascular endothelial growth factor (VEGF) pathway another promising target for gene suppression. ANGIOZYME, a hammerhead based ribozyme developed by Ribozyme Pharmaceuticals Inc., recognises and cleaves the mRNA of VEGF-R1 receptor [88]. Extensive preclinical studies have demonstrated no significant toxicities. The potential therapeutic use of this ribozyme against refractory solid tumors was assessed in several clinical trials as a monotherapy or in combination with other chemotherapy drugs [116,117]. ANGIOZYME was well tolerated with satisfactory pharmacokinetic variables. Results have provided the basis for subsequent clinical trials of this compound. ANGIOZYME is likely to be the first ribozyme drug to be approved.

Since it was first noticed that telomerase activity is present in the majority of malignant cells assuming a role in tumor progression and cell immortalization, the ribonucleoprotein complex of this enzyme was considered as a potential target for anti-cancer therapy. The telomerase RNA component and the mRNA of the reverse transcriptase subunit have been targeted and cleaved by hammerhead ribozymes with subsequent decrease of proliferating activity and induction of apoptosis in human melanoma cells [118], endometrial carcinoma cells [119], breast cell lines [120,121], colon and gastric carcinoma [122], and arrest of metastatic progression in a mouse melanoma model [123]. However, conflicting evidence, underline the need for unravelling the exact nature of telomerase action in tumorigenesis [122,124]. Hairpin ribozymes are less commonly used in the field of oncogene suppression. By use of a hairpin ribozyme gene library with randomized target recognition sequences in a mouse fibroblast model, telomerase reverse transcriptase (TERT) was identified as a target in order to suppress cell immortalization [125].

Drug resistance remains a serious limitation in the treatment of human cancers. Ribozymes, and most prevalently hammerhead motifs, have been used to downregulate mRNAs of drug resistance inducing genes in order to establish cellular sensitivity towards anti-cancer drugs. The ATP-binding cassette (ABC) transporter superfamily contains many such genes. ABC transporters are membrane proteins that translocate a wide variety of substrates across

extra- and intracellular membranes, including metabolic products and drugs [126]. Overexpression of certain ABC transporters occurs in cancer cell lines and tumors that are multi-drug resistant. MDR1 (ABCB1, also called P-glycoprotein) is a drug efflux pump that is responsible for decreased drug accumulation in multi-drug resistant cells. MDR1 interception by hammerhead ribozymes has increased chemotherapy sensitivity to several compounds (among them are vincristine, doxorubicin and cisplatin) in human lung cell lines [127], in colon-cancer cells [128,129], lymphoma cells [130], and hepatocellular carcinoma [131].

Hammerhead-mediated inhibition of ABCG2 (BCRP) resulted in sensitization to mitoxantrone and methotrexate in breast adenocarcinoma cell line [132], and in gastric carcinoma cell line [133].

MRP1 and MRP2 belong to the ABCC subfamily (alternative names are ABCC1 and ABCC2 respectively) and are known to confer to cell chemotherapy resistance. Specific cleavage of MRP1 mRNA by hammerhead ribozymes reverses nitrosourea and doxorubicin resistance of human glioblastoma cells [134]. MRP2 overexpressing human ovarian carcinoma, adenocortical carcinoma, and melanoma line were found cisplatin insensitive. When MRP2 was silenced by hammerhead ribozyme activity, platinum-resistant phenotype was reversed [135].

In a recent publication, a multitarget multiribozyme was constructed that targets the transcripts of the ABC transporters MDR1, ABCG2, and MRP2. This construct was tested in a series of cell lines: gastric carcinoma cell line, breast adenocarcinoma and ovarian carcinoma line. These three cell models overexpress MDR1, ABCG2, and MRP2 respectively. Individual ribozymes retained their catalytic activity when expressed as multitrancripts, and as a consequence complete reverse of resistance to daunorubicin, mitoxantrone and cisplatin was observed [136].

A different type of resistance against alkylating drugs such us alkylnitrosoureas and alkyltriazenes concerns the activity of cellular O^6methylguanine-DNA methyltransferase (MGMT). Functional inhibition of MGMT using a hammerhead ribozyme resulted in dramatic increase of mitozolomide sensitivity [137], while in a recent report MGMT inhibition by hammerhead technology resulted in nearly undetectable MGMT activity and BCNU sensitivity comparable to that of non resistant cells [138].

Other examples of genes implicated in chemotherapy drug tolerance that have been the subjects of ribozyme-based silencing are the following: gamma-glutamylcysteine synthetase (gamma-GCS, heavy chain) which apart from drug also lends resistance to ionizing radiation [139], human splicing factor SPF45 [140], survivin [141], heparan sulphate proteoglycan glypican-3 (GPC3) [142], and BRCA1 [143].

Inherited Genetic Diseases

Ribozyme technology has been implemented in the battle against various genetic diseases, which cause either insufficient production of essential proteins or accumulation of non-functional or cytotoxic proteins.

Osteogenesis imperfecta (OI) is a heritable dominant disorder of connective tissue caused by mutations in either of the α chains of type I collagen, which leads to fragile bones and an increased likelihood of fractures even on trivial trauma. There is an ongoing effort to selectively suppress the mutant allele alpha1 (I) collagen mRNA by hammerhead ribozymes without affecting the normal allele by direct mutation suppression [144] or polymorphism linked suppression [145]. The results so far concern *in vitro* cleavage and *in vivo* expression of the ribozyme transgene, and not yet a successful phenotype reversal to normal state [146-147].

Retinitis pigmentosa is an inherited autosomal dominant disease, which cause deterioration of the retina resulting in night blindness, tunnel vision and, eventually, loss of sight. At the molecular level, responsible for the phenotype is a substitution of histidine for proline at codon 23 (P23H) in the rhodopsin gene, resulting in photoreceptor cell death due to the synthesis of the abnormal gene product. The rational of therapeutic application of both hammerhead and hairpin ribozymes is again the specific cleavage of mutant alleles.

In a very promising series of reports, anti-P23H ribozymes administered by adeno-associated vectors in a mouse retinitis model, markedly slowed the rate of photoreceptor degeneration, even when delivered at a late developmental stage [40,149].

Other examples of potential realizations of ribozyme therapeutics in this field include Marfan syndrome [150], myotonic dystrophy [151], and sickle cell anaemia [152].

III. SELF-SPLICING INTRONS

RNA splicing is a crucial cellular process in the expression of many genes. During this process, the introns are excised from the pre-RNA transcripts and simultaneously the boundary exons are linked covalently. A large number of introns have been shown that catalyze their own splicing and on account of that, they are considered to be ribozymes. Based on their differential secondary structure and splicing mechanism, these self-splicing introns are distinguished in group I and group II. In both groups, chemical reactions follow a two-step transesterification mechanism using an in-line S_N2 nucleophilic substitution. In contrast with the small ribozymes, the attack is initiated by an external or a far distant internal nucleophile (group I and group II introns, respectively) and not by an adjacent internal one. At the second step the 5′ exon terminating in a 3′-OH attacks the 3′splice site, resulting in products with 5′-phosphate and 3′-OH ends [153,154]. Group I and group II ribozymes, which can be modified so as to act intermolecularly (*trans*-splicing), have been recently indicated as potential genetic tools in targeting viral RNAs, mutant genes and defective mRNAs.

Group I Introns and Applications

Group I introns, whose size vary from 200 nt to 1500 nt, are widely spread in almost all organisms such as prokaryotes and lower eukaryotes, as well as in organelles and T4 bacteriophages. These ribozymes were first isolated from *Tetrahymena thermophila* [155] and their self-splicing mechanism was described by Thomas Cech [156,157]. Group I introns contain four conserved sequence elements near the catalytic centre designated P, Q, R, S, and form ten

conserved double-stranded motifs (P1-P10) in their primary and secondary structure respectively [158]. The 5´ cleavage point is defined by the formation of a conserved U-G base pair upstream of the splice site. In the second step, which is essentially the reverse of the first chemical step, the 5´ exon attacks the 3´ splice site that is downstream of an invariant guanine residue resulting in the ligation of the two exons [156,159]. The presence of divalent cations, such as Mg^{2+}, is essential for catalysis [160].

Due to their ability to act *in trans*, group I introns are being considered as useful biotechnological tools aimed at both destroying or repairing mutant RNA molecules (Fig. (**3**)). An internal guide sequence, attached to the 5´ end of the ribozyme, recognizes a complementary region upstream of a nonsense or missense mutation of a defective transcript and splices it. The recombinant intron is also able to restore the genetic information through the ligation of the spliced mutant RNA with an exogenous exon-like attached to the intron's 3´ end, which provides the correct sequence of the gene (Fig. **3**) [9]. Sullenger and Cech were the first who managed to repair a truncated form of the *lacZ* mRNA *in vitro,* in *E. coli* cells as well as in mouse fibroblasts and to obtain a functional β-galactosidase by using the aforementioned strategy [161,162].

Recently, an efficient approach has been developed in order to treat cancerous cells via induction of wild-type p53 activity [163,164]. p53 is of great interest, because the development of many types of cancer have been correlated to several mutations in this gene. When bacteria and human osteosarcoma cells were cotransfected with two plasmids, the first containing a truncated form of p53 and the second the recombinant ribozymes carrying the missing fragment, repaired p53 transcript was detected in both cellular environments [164]. Further research was made in human colorectal carcinoma cell line SW480, in which endogenous mutant p53 protein is expressed. Transfection of these cells with a vector that contains the modified group I intron resulted in functional p53 production [164].

Similarly, *trans*-splicing ribozymes have been designed to restore genetic mutations which are involved in inherited disorders, such as sickle cell anemia and myotonic dystrophy. Sullenger *et al*. succeeded to convert sickle β-globin transcripts into mRNAs encoding the anti-sickling protein γ-globin in human erythrocyte precursors. Sequence analysis showed that the repaired RNA molecules maintain the ORF, but it still remains unknown whether these transcripts are translated into active fetal hemoglobin [165,166,167]. Additionally, myotonic dystrophy is caused by a CTG repeat expansion in the 3´ untranslated region of myotonic dystrophy protein kinase (DMPK) gene. DMPK mRNA has been targeted successfully, by appropriate *trans*-splicing group I introns both *in vitro* and in human fibroblasts expressing the mutant DMPK gene [168,169].

Group I introns, unlike hammerhead and hairpin ribozymes, are able to repair the defective mRNAs and this feature makes them important biotechnological tools. However, the group I intron design exhibits low specificity due to the short length of the internal guide sequence hybridizing with the target mRNA (6-8 nucleotides). This feature must be improved, before group I introns are used as therapeutic agents.

Group II Introns and Applications

The structurally most complicated naturally occurring ribozymes belong to group II introns and have been found in several eukaryotic organelles and prokaryotes, where their size range from 300 nt to 3000 nt. Group II introns contain six helical domains (I-VI) emerging from a central core [170], but only domains I and V have been identified to play a major role for catalysis. Domain I contains the specific sequences EBS1 and EBS2 (Exon Binding Sequence 1 and 2), which interact with IBS1 and IBS2 (Intron Binding Sequence 1 and 2). This interaction is indispensable for the 5´ exon recognition. As far as domain V is concerned, it has been reported to harbour the reaction center of the ribozyme [171,172]. As described for most ribozymes group II introns folding and catalytic activity requires Mg^{2+} ions [173,174].

Apart from the fact that group II introns can mature RNA molecules, it has also been proven that they can be transposed and inserted into double-stranded DNA target sites, by the expression of an intron-encoded protein (IEP), which acts as a reverse transcriptase, maturase and DNA endonuclease. Subsequently, group II introns libraries with randomized EBS1 and EBS2 sequences are constructed and screened by the target molecule in order to select the most specific ribozyme. Group II introns' advantage against group I introns is their higher specificity, which results from the recognition of a 14 nt region of DNA juxtaposed to the fixed sites of IEP interaction [175].

Only preliminary studies have been performed so as to achieve gene silencing and repairing of mutant genes through group II usage. More specifically, a mobile group II intron from *Lactococcus lactis* has been integrated to extrachromosomal HIV pro-virus DNA and human CCR5 gene in the cellular environments of *E.coli*, human embryonic kidney and CEM T cells. This integration disrupts the genes of CCR5 and HIV pol, clearly suggesting a therapeutic use of group II introns in coping with HIV infection [175]. Furthermore, group II introns are able to insert efficiently into chromosomal genes in bacteria [176].

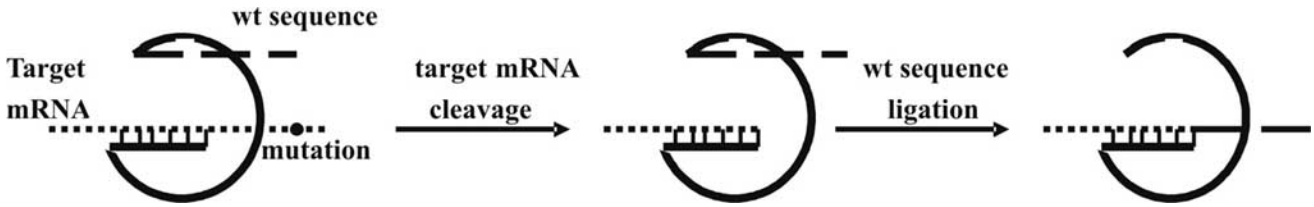

Fig. (3). Simplified structure and mode of action of a group I intron.

These results demonstrate that mobile group II introns could be used to destroy harmful DNA sequences in human cells. Finally, modified ribozymes were engineered to repair mutant *lacZ* and human β-globin gene by wild type exon insertion in a bacterial cell system, and in both cases the desired ORF was retained. This ribozyme can distinguish the target sequence from other similar or homologous ones [177]. Mobile group II introns are considered to be promising therapeutical agents for future applications in the context of gene therapy.

IV. RNA INTERFERENCE (RNAi)

The Mechanism of RNA Interference

RNA interference (RNAi) is an endogenous cellular defense mechanism. The physiological role of RNAi process is the host cell protection from invasion by exogenous nucleic acids introduced by mobile genetic elements, such as viruses and transposons [178]. It was first observed in plants in the late 1980's but the molecular mechanism remained elusive until the late 1990's. The mechanism relies on the recognition of dangerous double-stranded RNA molecules (dsRNA) and subsequent enzymatic cleavage of any mRNA transcript with homology to these dsRNAs. RNAi mechanism includes the segmentation of dsRNAs of various lengths, produced by the cell or introduced into the cell, by the dsRNA endoribonuclease Dicer into ~21nt small interfering RNAs (siRNAs). RNAi can also be induced in mammalian cells by the introduction of chemically or enzymatically synthesized double-stranded small interfering RNAs, or by plasmid and viral vector systems that express double-stranded short hairpin RNAs (shRNAs) that are subsequently processed to siRNAs by the cellular machinery. These siRNAs in turn associate with an RNAi-inducing silencing complex (RISC) and direct the destruction of mRNA molecules that are complementary to the antisense siRNA strand. RISC cleaves the target mRNA in the middle of the complementary region, thus silencing gene expression (Fig. (**4**)) [179-181]. Similar defense functions are also present in mammals with the endogenous microRNAs that participate in the process that regulates the expression of genes involved in a variety of cellular processes such as proliferation, apoptosis and differentiation [182].

Potential Use of RNA Interference-Based Therapies

Gene regulation and silencing using the RNA interference could prove out a powerful tool for sequence-specific therapeutics against a wide variety of diseases, since various human diseases root in the inappropriate expression of specific genes. Much of the success of RNAi as a therapeutic tool is due to the fact that the enzymatic machinery required to process siRNAs is endogenous, ubiquitously expressed and in addition, it can be stimulated by exogenous RNAs to direct sequence-specific gene silencing [183].

The ability to utilize this native pathway to create a new class of innovative medicines has been recognized as one of the most exciting biotechnological advances. Therapeutic approaches based upon RNAi are postulated to have a strong combination of inherent benefits. It is possible to design siRNA drugs for every mRNA and by this means to overcome the limitation of the conventional medicines that

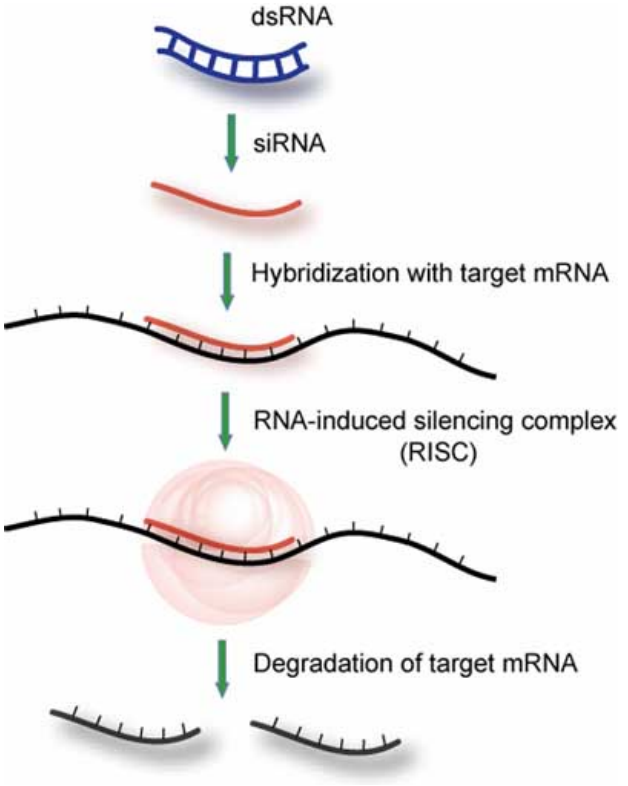

Fig. (4). siRNA-mediated mRNA degradation.

can only target specific protein classes. For example, although sequencing data from the human genome have revealed the key disease-causing genes and the corresponding protein targets, it has been proven difficult for several of them to serve as targets for small molecule inhibitors or antibodies. In contrast, siRNA-based drugs can be widely used, as they prevent the primitive gene expression, providing greater efficacy in disease control. Bioinformatic tools in combination with the available sequencing data give the opportunity to design drugs with complementary specificity to the target mRNA sequences [184].

RNAi is among the most specific methods available for the functional inactivation of genes and, when appropriately used, a single nucleotide mismatch can be sufficient for discrimination between the target and the off-target sequence. The usage of siRNAs to down-regulate only the mutant version of a gene has been shown to have highly specific effects on tumor cells, while normal cells remain untouched [185]. RNAi-mediated inhibition of gene expression is potent and versatile enough in comparison with other silencing techniques. As a natural strategy requires lower concentrations of oligonucleotides in order to inactivate a specific gene at the mRNA level and can target multiple sequences, within an individual gene or a group of genes, which leads to a greater percentage of target gene silencing [186,187].

Stability of siRNAs

Therapeutics based on RNAi are promising for the cure of several diseases but before the clinical trials of such drugs

there are some points that have to be extensively clarified. Since siRNAs are naturally degraded, their effect in the cells is transient and therefore their gene-silencing activity should be prolonged. Modifications to the sugar moieties, phosphate linkage, bases and even 5′ caps for the RNA ends can be designed to protect these molecules from nuclease degradation. Chemical modifications, such as the use of phosphorothiate in the 3′ terminal linkage and 2′ modification of specific riboses, help siRNAs to escape from exonucleases and endonucleases, respectively. Another type of modified siRNAs, boranophosphate siRNAs, seems to be more effective at silencing than phosphothioate siRNAs and more resistant to nucleases than the unmodified siRNAs [188]. Similarly, siRNAs containing 2′ O- methyl and 2′-fluoro nucleotides display enhanced stability and increased potency [189]. The enhancement of the siRNAs' stability *in vivo* will result in more sustained silencing effects.

Cellular Uptake of siRNAs

Once the siRNAs stability is obtained, specific focus on cellular uptake must be given. The first hurdle that siRNAs have to overcome due to their anionic character is the cell membrane. Moreover, as it is expected, cells do not favor RNA uptake because this event usually signifies a viral infection. Therefore, when unmodified RNA is injected into the bloodstream it is rapidly excreted by the kidneys or gets degraded [190]. Successful RNAi therapeutics must use strategies that reduce renal filtration. This can be achieved by the adjustment of the siRNA effective size (i.e. conjugation with polyethylene glycol, lipid encapsulation or modifications that serve the binding to plasma proteins) [191]. The *in vivo* retention time of the siRNAs can be increased by complexion with lipids or protein carriers to limit renal filtration. In principle, complexes can be designed to enhance the rate of uptake into the cell and, potentially, direct the siRNAs to specific cell types. Coupling of siRNAs to basic peptides has been reported to facilitate the transport of siRNAs across the cell membrane [192].

There are two ways to introduce siRNAs into the cells. The first is the direct delivery and the second is insertion through DNA encoding short hairpin RNA (shRNA) expression cassettes. The latter will be expressed and subsequently processed to siRNAs by the cellular machinery [193]. The first method gives the opportunity to control the amount and purity of chemically synthesized and characterized siRNAs and the ability to introduce modifications into the siRNAs in order to label them or to enhance their efficacy.

The second method has the important advantage that cellular exposure can occur for a prolonged period of time [194]. It is preferable because the DNA vectors are more stable in the cell environment and they could allow continual expression of the siRNAs and subsequently, gene silencing. Moreover, this approach possesses several additional advantages compared with administration of chemically synthesized siRNA: i) regulatory elements could be added to the promoter region of the plasmid such that tissue-specific silencing occurs with a systemically administered plasmid; ii) permanent gene 'knock-down' cell lines can be established for *in vitro* work, or for generation of 'knock-down' animals through cloning.

Direct Administration of siRNAs

The most significant obstacle for RNAi-based therapy is the efficient and effective delivery of RNAi reagents in patients. A number of strategies have been developed that allow siRNAs and shRNAs to be delivered effectively in animals. As far as direct administration of synthetic siRNAs is concerned, multiple methods have been shown to be successful. The hybrodynamic strategy relies on the intravenous injection of siRNAs in a large volume of saline solution, which works by creating a back-flow in the venal system that forces the siRNA solution into several organs (mainly the liver, but also kidneys and lung with lesser efficiency) [195]. *In vivo* delivery can be also achieved by injecting smaller volumes of siRNAs that are packaged in cationic liposomes. When siRNAs are administered intravenously using this strategy, silencing is primarily seen in highly perfused tissues, such as the lung, liver, and spleen [196]. Local delivery of siRNAs has been shown to be successful in the central nervous system [197]. Finally, electroporation of siRNAs directly into target tissues and organs has lead to efficient gene silencing [198,199]. Electroporation has been used to deliver siRNAs into the brain [200], eyes [201], muscles [202], and skin [203] of rodents. Topical gels have also been used to deliver siRNAs to cells and could open the way for dermatological applications, as well as the treatment of cervical cancer [204].

Systemic Delivery of siRNAs

For the systemic delivery of RNAi-based drugs, several parameters must be taken into concideration. The cell and tissue specific delivery of siRNAs and shRNAs has been achieved by conjugating RNAs to membrane-permeable peptides and by incorporating specific binding reagents such as monoclonal antibodies into liposomes used to encapsulate siRNAs [205,206]. Alternatively, siRNAs can be entraped into PEG-immunoliposomes (PILs) which are covered with receptor-specific monoclonal antibodies or other targeting proteins, for tissue-specific delivery [205]. To improve the delivery of siRNA into human liver cells without transfection agents, lipophilic siRNAs conjugated with derivatives of cholesterol, lithocholic acid, or lauric acid can be synthesized [207]. By conjugating cholesterol to the 3′-end of the sense strand of siRNA (by means of a pyrrolidine linker), the pharmacological properties of siRNA molecules were improved [208]. Besides being more resistant to nuclease degradation, the cholesterol attachment stabilized the siRNA molecules in the blood by increasing binding to human serum albumin and increased uptake of siRNA molecules by the liver.

The shRNA encoding expression cassettes can be introduced into the cells through plasmid transfection or viral transduction, which seem to work similarly. To obtain efficient and prolonged gene silencing using RNAi in cells and tissues, a variety of viral vectors have been developed to deliver siRNAs both *in vitro* and *in vivo*. Retrovirus-based vectors that permit stable introduction of genetic material into cycling cells [209] have been engineered to express shRNAs and to trigger RNAi in transformed cells, as well as in primary cells [210,211]. They have been also used to create "knockdown" tissues in mice, since they can be expressed in certain adult stem cells, notably hematopoietic

stem cells. Moreover, retroviruses for RNAi could potentially be applied for *ex vivo* cellular manipulations, including those of dendritic cells for the modulation of immune responses [212]. However, the use of these vectors may be associated with a risk of insertional mutagenesis and should be carefully evaluated [213]. In addition, wide-ranging applications of RNAi have been reported using recombinant lentiviral vectors, because they permit infection of noncycling and postmitotic cells such as neurons [210]. Lentiviral transduction has the advantage of stable integration into the genome and therefore making the silencing process more efficient [214].

Highly effective siRNA delivery systems have also been created that are based on adenoviruses and adenovirus-associated viruses (AAV) [215-217]. Adenoviruses do not integrate into the genome and tend to induce strong immune responses, which may limit their use in some circumstances. On the other hand, AAV does not cause disease in humans [218] and can integrate into the genome of infected cells at a defined location, eliminating the chance of a mutagenic effect. [219,220]. Effective gene silencing in the liver and the central nervous system, mediated by AAV-based vectors, has been demonstrated following systemic or tissue-specific injection of viral particles [221,222]. In general, the use of viral vectors for the systemic delivery is not strongly recommended, as the process lacks satisfactory tissue or organ specificity, and engulfs the danger of malignant transformation induced by those vectors [185,210].

Tissue-Specific Delivery of siRNAs

In order to obtain specificity during the delivery of siRNAs to organs and tissues it has been proposed the encapsulation of siRNAs into liposomes or lipoplexes that contain on their surface adducts that target specific cell receptors. Such a method was implemented for the delivery of siRNAs designed to silence an oncogene expressed in Ewing sarcoma. The siRNAs inhibited human tumor growth in mice when they were packaged in a cyclodextrin polymer and targeted through the attachment of transferring to polymer. Finally, the antibody-mediated *in vivo* delivery of siRNAs has been successfully applied in an attempt to silence the HIV-1 envelope and capsid gene *gag* [223].

Evaluation of the si-RNAs Use Before Clinical Trials

Once the siRNAs-delivery technique is defined and before the clinical trials, there must be an evaluation between the benefits and the risks. During the RNAi-based therapeutics the cells will be exposed to exogenous reagents, such as the siRNAs and the vehicles that will be used for the delivery, and there is the possibility to disturb normal cell functions. Moreover, it must be ensured that there will not be off-target effects on the expression of other genes with relevant homology to the targeted one. There are situations where mismatches between the siRNA and target sequence can be tolerated [224]. For these reasons, the parameters that determine the minimum level of homology required for siRNA-mediated silencing must be defined. It is interesting that off-target effects are not observed when dsRNAs are used in primitive organisms.

The immune system of mammals seems to get disturbed by the RNAi mechanism that may trigger an antiviral interferon response mediated by the protein kinase regulated by RNA (PKR). The interferon response causes non-specific gene silencing and apoptosis in mammalian cells and may lead to artifacts in correlation with the prospective gene silencing [225]. A second major concern is the fact that siRNAs and shRNAs can activate dendritic cells and other cells of the immune system through a much more specific and restricted class of receptors, the Toll-like receptors (TLRs), that can recognize foreign nucleic acids including dsRNAs [226,227]. Thus, RNAi reagents may trigger adverse immune responses *in vivo.*

It must be also studied whether the endogenous RNAi mechanism is saturated when the "guests" siRNAs are introduced into the cells. If these siRNAs utilize all the available DICER and RISC enzymes, then there will not be RNAi activity as in normal conditions when the cell will have to face a "threat". Therefore, the availability of the RNAi-mechanism reagents must be defined and the potency of the system to cope with both its natural role and the exogenously induced gene silencing must be evaluated.

Another possible problem that can rise from the usage of RNAi-based therapeutics especially against virus infections is the resistance obtainment. To combat resistance, multiple sequences per target and multiple targets per viral genome have to be targeted. Another form of resistance is the fact that not all sequences can be targeted by siRNAs. This is likely due to a lack of accessibility of the RNA sequence, either hidden by RNA-binding proteins or by complex secondary structures. Usage of computing software can help to predict the accessibility of RNA binding sites and minimize the targeting of inaccessible sites [228]. Lastly, cells may also develop resistance to RNAi through loss of genes essential for RISC complex formation or selection of suppressors that inhibit degradation. Cymbidium ringspot virus is resistant to RNAi via production of p19, a protein that inhibits RNAi by sequestering dsRNAs [229]. Although these forms of resistance are largely hypothetical in humans, appropriate selective pressure could lead to similar problems.

Finally, the fact that RNAi does not work well in all cell types may be inhibitory for the corresponding therapeutics. It has been reported that in neurons of *C. elegans*, the silencing process is not applicable as a dsRNA specific RNase is expressed in this tissue [230]. A deeper understanding of the mechanisms negatively regulating RNAi may contribute to ways of artificially increasing the efficiency of the RNAi process. Overall, for the precise and constructive practice of RNAi-based therapeutics, there must be persistent research on this emerging field in order to reveal all the special characteristics and key elements for the improvement of the silencing technique.

Applications of RNA Interfence-Based Therapeutics

One of the earliest proposed therapeutic uses of RNAi was to inhibit viral infection. Many genes from important human viral pathogens, including HIV, HBV, HCV, influenza virus, and SARS coronavirus, have been silenced by RNAi, causing inhibition of viral replication *in vitro* and in mouse models of viral infection.

Initial *in vivo* experiments with HBV-specific siRNAs (or shRNA expression vectors) introduced to the mouse liver

showed that the induction of HBV gene expression and replication can be inhibited, in the cases that the siRNAs are administered simultaneously or after the HBV infection [231,232].

In the case of HIV, virus production was inhibited by effective silencing of the primary HIV cellular receptors CD4 and CCR5, the viral structural Gag protein or the green fluorescence protein substituted for the Nef regulatory protein. The two cellular receptors are appealing antiviral targets, because siRNAs that are targeted directly to the viral genome could lead to the generation of viral escape mutants [233,234].

Hepatitis C virus (HCV) was recently shown to be sensitive to RNAi. Addition of siRNA to silence various portions of the hepatitis C virus genome led to a 98% reduction in detectable virally infected cells [235]. Moreover, the introduction of siRNAs targeting the 5´UTR of the HCV replicon, resulted in 80% suppression of HCV replication [236].

Likewise, inhibition of influenza virus [237], coxsackievirus B3 [238], SARS-virus [239] and respiratory syncytial virus (RSV) [240] infections have been inhibited by siRNAs delivered after the establishment of infection in mice.

Over-expression of genes that promote cell proliferation, or inhibit apoptosis (oncogenes) and inactivation of genes that inhibit cell proliferation or induce apoptosis (oncosuppressor genes), are responsible for neoplastic transformation. The appeal of RNAi-based silencing of the genes that are involved in cancer is the promise of cancer cells-specific death, in the absence of collateral non-neoplastic cell damage, which follows conventional chemotherapy.

Initial *in vitro* studies have demonstrated effective silencing of a wide variety of mutated oncogenes such as K-Ras [241], mutated p53 [242], Her2/neu [243], and bcr-abl [244].

The fusion gene M-BCR/ABL, which leads to chronic myeloid leukemia (CML), and the oncogenic K-RASV12 allele, which constitutively activates Ras leading to pancreatic and colon cancer, were succesfully silenced by sequence-spesific siRNAs [245,185]. The induced silencing leads to the loss of anchorage-independent growth and tumorigenicity and can reverse the oncogenic phenotype of cancer cells. The cancer cell's survival, under anchorage independent conditions, is also remotely controlled by the carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6), which is widely over-expressed in human gastrointestinal cancer. Administration of CEACAM6-specific siRNAs suppressed primary tumor growth and decreased the proliferating cell index, impaired angiogenesis and increased apoptosis in the xenografted tumors [246].

Another optimal candidate of anticancer strategies is Bcl-2, which is over-expressed in most cancers and makes the cancer cells resistant to programmed cell death. [247]. The oncogenes' silencing may slow the tumor growth but it does not reduce the mass of the cancer cells. Moreover, minifying the proliferation rates of cancer cells can evolve to eliminated effectiveness of traditional therapies that preferentially target actively dividing cells. Thus, the need for synergetic activity of conventional therapeutics and RNAi-based drugs ruled the definition of new targets. For example, silencing of the anti-apoptotic bcl-2 gene sensitizes cells to chemotherapy agents, such as etopodise and daunorubicin [248,249]. Likewise, combining RNAi and conventional chemotherapy can result effectively in the treatment of patients that have developed multidrug resistance, due to the overexpression of the multidrug resistance gene (MDR1) [250], RNAi-mediated suppression of MDR1 has been shown to re-sensitise cells to the effects of chemo-therapy [251,252].

Finally, another cancer treatment approach is the indirect control of tumor growth by inhibiting infiltration, spread and metastasis of cancer cells. For example, RNAi-mediated inhibition of the chemokine receptor CXCR4, which promotes metastasis to organs abundant in CXCR4 ligand in the case of breast cancer, results in reduced cell invasion *in vitro* and blocks the breast cancer metastasis in animal models [253].

As a more general approach, genes involved in angiogenesis are potential anti-cancer treatment targets, as new blood vessels are required for tumor growth. For this purpose silencing of VEGF and its receptor was tested for anti-tumor effects and resulted to blocked angiogenesis and limited tumor growth [254-256].

Over-expression of mutated genes was proven to be the primitive cause of several neurodegenerative diseases raising the possibility to use RNAi-based therapeutics for their treatment. Alzheimer's disease, Huntington's disease (HD), fragile X syndrome and amyotrophic lateral sclerosis (ALS), are some of the prominent neurological diseases susceptible to RNAi-based therapies.

Amyotrophic lateral sclerosis (ALS) is caused by single nucleotide mutations in the Cu^{2+}-Zn^{2+} superoxide dismutase (SOD1) gene. The optimal therapeutic strategy should target only the mutant SOD1, as the wild type performs important functions. This single nucleotide specificity is offered by the RNAi silencing [257].

Alzheimer's disease is caused by an increase in b-amyloid production, which requires cleavage by b-secretase (BACE1), an enzyme that is up regulated in the brain of Alzheimer's patients. Silencing of BACE1 in mouse models showed that there was no generation of b-amyloid peptides and obvious developmental abnormalities, indicating that this gene is a preferable target for the RNAi based Alzeheimer's treatment [258].

Huntington's disease (HD) results from polyglutamine repeat expansion (CAG codon, Q) in exon 1 of huntingtin that leads to toxic protein products. RNAi directed against mutated human huntingtin reduced its expression in cell culture and in HD mouse brain and improved behavioral and neuropathological abnormalities associated with this disease [259].

Finally, RNAi therapeutics could be usefull for the treatment of diseases that rely on the activation of innate cellular processes. In the case of rheumatoid arthritis (RA), for example, the TNFa, a proinflammatory cytokine, is involved in its chronic pathogenesis. Thus, siRNAs against TNFa might provide effective means of reducing inflammation in RA patients [260].

V. ANTISENSE-BASED THERAPEUTICS

The inherent simplicity of the antisense–mediated gene silencing raised considerable interest in the potential of using antisense deoxy-oligonucleotides (ASOs) as RNA-mediated therapeutic agents. ASOs are single strands of short nucleotide sequences (18–21 oligomers) that bind to complementary targeted mRNA molecules in a sequence-specific manner thus blocking translation (Fig. (**5**)). This process can activate endogenous nucleases, such as RNase H, which cleaves the RNA strand of a heteroduplex RNA–DNA complex and release the oligonucleotide. In addition, some ASOs exhibit non-catalytic antisense effects, causing modulation of RNA splicing [261].

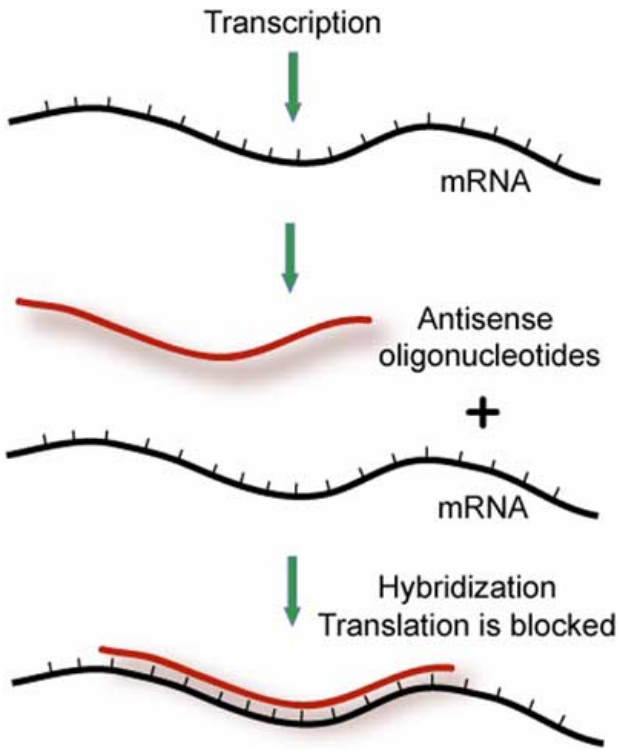

Fig. (5). Antisense oligonucleotide blocks mRNA transcription by hybridization.

As expected, the cellular uptake of ASOs is difficult due to their polyanionic properties. To overcome this barrier several modifications on the backbone of these molecules have been tested. Modifications in position 2 and alterations in the sugar-phosphate backbone have lead to the synthesis of ASOs with increased cellular uptake and resistance to nucleases [262,263].

The best known ASOs are the phosphorothioate class which are formed by the substitution of the nonbridging oxygen atoms in the phosphate group with a sulphur atom, resulting in ASOs that are negatively charged, highly soluble and more resistant to endonucleases with greater hybridisation ability for target RNA [264].

Phosphorothioates are polyanions and they can ineract with proteins containing polycation binding sites. Such proteins include a large number of heparin binding proteins [265,266], such as bFGF, PDGF, VEGF, EGF-R [267], CD4, gp120 [268], Mac-1 [269], laminin, fibronectin, and many others, and their non-specific binding with ASOs can influence their pharmacology and toxicity [270].

Moreover, in animals, some ASOs interact with the intrinsic clotting cascade [271] and activate the alternate complement pathway [272]. According to these observations there is the possibility that a biological effect of antisense-based therapeutics may be produced not by the antisense mechanism but due to a complex combination of non–sequence specific effects.

The only antisense drug that has received FDA approval so far is Vitravene, from Isis Pharmaceuticals in Carlsbad, California. Vitravene is a small antisense single-stranded DNA molecule with phosphothioate backbone and is used to treat cytomegalovirus infections in the eye for patients with HIV. Recently, another promising antisense-based drug is Genasense that targets Bcl-2, a protein expressed in high levels in cancer cells, which is thought to protect them from standard chemotherapy. The FDA is currently reviewing an application for Genasense, as there are promising results in the treatment of malignant melanoma.

VI. RNASE P

Ribonuclease P (RNase P) is an essential endonuclease that acts early in the tRNA biogenesis pathway catalyzing cleavage of the leader sequence of precursor tRNAs (pre-tRNAs) and generating the mature 5' end of tRNAs. RNase P activities have been identified in bacteria, archaea, and eucarya, as well as in organelles. Most forms of RNase P are ribonucleoproteins, i.e., they consist of an essential RNA and protein subunit(s); the composition of the mitochondrial enzyme remains to be elucidated. Bacterial RNase P RNA was one of the first catalytic RNAs identified and the first that acts as a multiple turnover enzyme. RNase P and the ribosome are so far the only two ribozymes known to be conserved in all kingdoms of life [273,274]. The RNA component of bacterial RNase P can catalyse pre-tRNA cleavage in the absence of protein subunit *in vitro* and consists of a specificity domain and a catalytic domain. Bacterial RNase P can be sub-divided in two major types (A and B) on the basis of their sequence characteristics [275]. The best characterized RNase P RNA molecules come from two bacteria (*Escherichia coli* and *Bacillus subtilis*) which are paradigms of the A and B type respectively. The RNA component of bacterial RNase P consists of 350-450 nucleotides, whereas the protein component is a small basic protein of about 120 aminoacids [274]. The small protein subunit, an extremely basic protein, binds near the catalytic core of RNase P RNA and directly interacts with the pre-tRNA substrates facilitating pre-tRNA recognition and binding, and modulating RNase P RNA structure [276].

Studies on human holoenzyme showed that one RNA subunit (H1 RNA) and at least ten essential proteins with molecular weights ranging from 14 to 115 kDa [277] contribute to the total mass of RNase P. H1 RNA has not been shown to possess catalytic properties under any conditions in the absence of the protein complement. Extensive deproteinization of eukaryotic enzymes leads to

loss of enzymatic activity with one exception. Recently, we reported a new catalytic activity subsequent to extensive deproteinization of *Dictyostelium discoideum* RNase P; the proteinase K/phenol/SDS treated enzyme cleaves tRNA precursors several nucleotides upstream of the cleavage site of RNase P and liberates products with 5´ hydroxyl ends. It seems that this activity is associated with two RNA molecules co-purifying with *D. discoideum* RNase P activity [278].

The application of RNase P in gene inactivation was achieved by using two strategies. A guide sequence (GS), complementary to an RNA target, is covalently attached to the 3´ end of M1 RNA (M1GS) [279]. This modification converts the M1GS from a structure specific ribozyme to a sequence specific one. The concept of this modification is that when the GS binds to the target RNA a structure equivalent to the top portion of a precursor tRNA (the minimal requirement for substrate recognition of M1 RNA) can be formed. Then the M1GS RNA hydrolyzes successfully the target RNA (Fig. (**6A**)). The other strategy takes advantage of the endogenous RNase P activity, which can be used to digest cellular or viral mRNAs. This can be achieved with the help of an external guide sequence (EGS). EGS must be designed in such way to form a structure resembling to a portion of the natural tRNA substrates of the enzyme when it hybridizes to the target RNA [279]. This leads to specific cleavage of the target RNA by RNase P in the nucleus (Fig. (**6B**)).

The lack of effective antiviral and anti-cancer drugs led to a substantial effort for the development of new therapies based on both of the above mentioned strategies. M1GS ribozymes have been designed to cleave various targets including oncogenic mRNA, the herpes simplex virus (HSV) and cytomegalovirus (HCMV) essential mRNAs. EGS have been designed for RNase P-mediated inhibition of human immunodeficient virus (HIV), influenza virus, HSV, HCMV and Kaposi's sarcoma (KS)-associated herpesvirus (KSHV). Also, effort has been made to apply the EGS technology to the therapy of asthma and other atopic diseases by developing EGS to direct RNase P mediated cleavage against interleukin-4 receptor α mRNA.

M1 RNA ribozyme has been proved to be particularly useful for the inhibition of chimeric gene products created by chromosomal abnormalities. A model target is a well-characterized example in the hematopoietic system that involves the rearrangement of the BCR and ABL genes in Philadelphia chromosome positive ($Ph1^+$) chronic myelogenous leukemia and acute lymphoblastic leukemias. This translocation results in the formation of chimeric BCR-ABL oncogenes. Using M1GS RNA with guide sequence that recognize the oncogenic messengers at the fusion point (otherwise, normal mRNA that shares part of the chimeric RNA sequence will also be cleaved by the M1-GS RNA, with resultant damage to host cells) the ribozyme can effectively inhibit the expression of the BCR-ABL fusion transcripts found in the cancerous cells of leukemia patients [280].

When an M1GS ribozyme, designed to cleave the mRNA encoding the major transcriptional activator ICP4 of HSV-1, was expressed in human cells infected with HSV-1, not only caused a reduction of about 80% in the expression of ICP4, but also decreased a 1000-fold the viral growth [281,282]. Similarly, an M1GS ribozyme that targets the overlapping region of HCMV immediate early gene 1 and 2 (IE1/IE2), reduces IE1/IE2 expression by 85% and inhibit HCMV growth by 150 fold [283]. Recently, in an excellent study, a selection system for generating M1GS RNA variants has been developed that efficiently cleave a model substrate derived from the HSV-1 thymidin kinase (TK) mRNA *in vitro* [284].When cell lines that expressed a M1GS variant targeting HSV-1 TK mRNA were infected with HSV-1, a reduction of up to 99% of TK expression was observed as compared to a reduction of 70% in cells that expressed the wild type ribozyme [284]. Also, ribozyme variants designed to target HSV-1 ICP4 and HCMV IE1/IE2 mRNAs caused a reduction of 90% and 97% in the expression level of ICP4 and IE1 and IE2 and as well as a reduction of 4000-fold and 3000-fold in viral growth respectively [285,286]. Similarly, when a ribozyme variant, bearing point mutation at nucleotides positions 80 and 188 of M1 RNA, designed to target the overlapping region of HCMV IE1 and EI2 mRNAs was used, the rate of catalytic cleavage was increased, and as well as the substrate binding of the ribozyme was enhanced. Moreover, in cells where this ribozyme variant was expressed a 99% reduction in the expression of IE1 and IE2 and a reduction of 10,000-fold in HCMV was observed

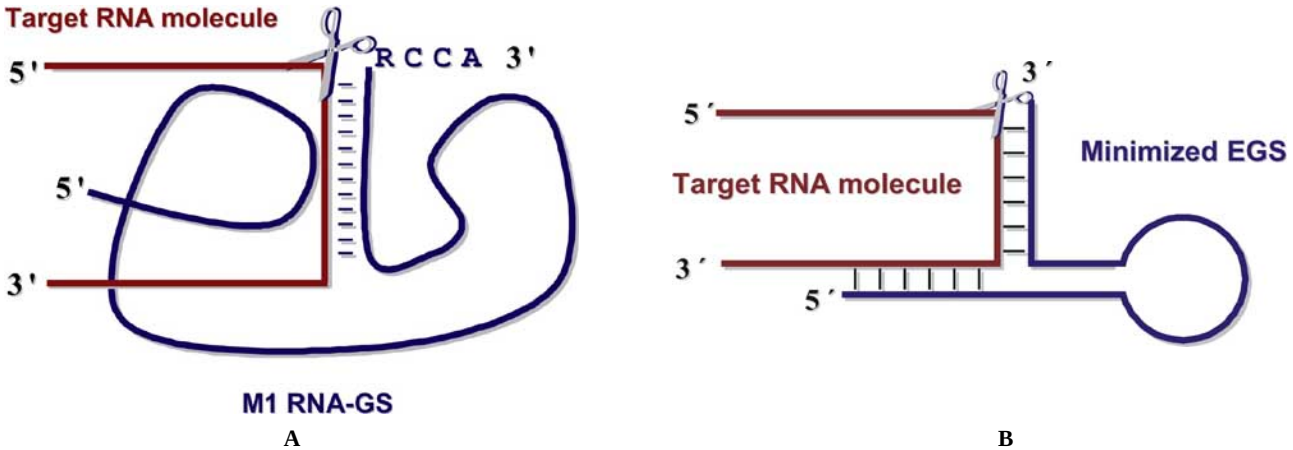

Fig. (6). M1 RNA-GS (**A**) and a minimized EGS (**B**) bound to a target RNA molecule.

[287]. Also, an M1GS ribozyme constructed to target the over-lapping region of the mRNAs coding for HCMV capsid assembly protein (AP) and protease (PR) cleaves the target mRNA sequence *in vitro*, and leads to a significant inhibition of the expression level of viral AP and PR by 80-82% and inhibited viral growth by 2000-folf in cells expressing the ribozyme [288].

The EGS-based technology is an attractive approach for gene inactivation because it utilizes endogenous RNase P to generate highly efficient and specific cleavage of the target RNA. Ma and colleagues [289] reported that EGSs designed to target the 3´ region of the PKC-α mRNA downregulate PKC-α protein and mRNA expression in T24 human bladder carcinoma cells, through activation of the endogenous RNase P. EGS can perform this function in living cells in the absence of RNase H mediated irrelevant cleavage observed with the antisense nucleotide approach. Plehn-Dujowich and Altman [290] showed that when mouse cells transfected with synthetic genes that constitutively expressed EGSs directed against polymerase subunit 2 (PB2) and nucleocapsid (NP) influenza virus mRNAs were infected with the virus, the subsequent RNase P mediated cleavage of mRNAs caused an inhibition of both protein synthesis and viral particle production by 90-100%. Fenyong Liu and his collaborators have developed several EGS RNAs which efficiently direct human RNase P against viral proteins causing the inhibition of their expression level and as well as the viral growth. EGS RNAs derived from a natural tRNA efficiently directed human RNase P to cleave the mRNA sequence enconding the thimidine kinase (TK) of HSV-1 *in vitro* [291,292]. A reduction of 75-80% in the TM RNA and protein expression was observed in HSV-1 infected cells expressing the EGS RNAs. In a recent study, human cells expressing EGS RNA constructed to target the overlapping mRNA region of two HCMV capsid proteins, the capsid scaffolding protein (CSP) and assembling, a reduction of 75-80% in the mRNA and protein expression levels and a reduction of 800-fold in viral growth were observed [293]. In an earlier study, Liu and collaborators administrated exogenously into human cells infected with HCMV a chemically synthesized DNA-based EGS molecule to target the mRNA coding for the protease of the virus. The EGS efficiently directed human RNase P to cleave the target RNA. A reduction of 80-90% in the protease expression was achieved and a reduction of about 300-fold in HCMV growth was observed [294]. Furthermore, exogenous administration of 2´-*O*-methyl-modified EGSs designed to target the mRNA encoding KSHV immediate-early transcription activator Rta into human cells infected with the virus, caused a reduction of 90% in Rta expression and a reduction of about 150-fold in viral growth [295]. 2´-*O*-methyl modified oligonucleotides may represent a class of antiviral compounds that can be administrated directly for gene targeting applications because can be easily synthesized chemically and are extremely resistant to degradation by various exonucleases and edonucleases.

The limitations of combination antiviral drugs therapies for human immunodeficiency virus (HIV-1) have lead to the design of gene inactivation as an alternative therapeutic approach of HIV infection. The most common approaches to gene inactivation for the treatment of HIV infection have mainly involved antisense technologies or ribozymes. Recently, effort has been made to apply the EGS-based technology for the inactivation of the HIV growth. An EGS that specifically recognizes and hybridizes to the U5 region of the 5´ leader sequence of HIV-1 leads to the successful cleavage at this region by the endogenous RNase P and degradation of the genome due to the removal of the protective 5´ cap. Heterogeneous cultures of $CD4^+$ T cells expressing the U5 EGS were protected from cross-clade HIV-1 infection and cytopathology with no loss of CD4 expression by RNase P mediated inhibition. It has been suggested that possibly the U5 EGS could inhibit viral infection following viral entry into the cytoplasm and before generation of proviral DNA. [296,297]. In a resent study Barnor and collaborators [298] designed an EGS of only 12 nucleotides long, which has similar inhibitory effect on HIV-1 expression to those containing the T-stem and loop of the tRNA precursor.

EGS targeting cytokine receptors is a novel approach to therapeutics. Dreyfus and coworkers [299] designed an EGS which directs efficient RNase P mediated cleavage of mRNA for the human IL-4r mRNA *in vitro*. Moreover, in lymphoblastoid cells expressing the EGS the inactivation of basal IL-4 signaling was evident.

The RNase P complex may offer an excellent alternative to conventional gene interference therapies for the treatment of infectious diseases and human malignancies. EGS-mediated RNA inactivation of targeted mRNA *in vivo* can be considerably more effective than gene inactivation by conventional antisense oligonucleotides. Moreover, in RNAi based technology the RNA induced silencing complex (RISC) must be induced. It is not known yet if RISC is present or it can be induced in all cell types. Additionally, as it has been mentioned before, the availability and the potency of the RNAi mechanism to serve both its natural role and the exogenously induced gene silencing remain to be evaluated. On the other hand large amounts of RNase P are present in all cell types at all times, ensuring the inactivation of the expression of a specific gene.

The RNA-mediated technology offers valuable tools for therapeutic applications. It is evident that a combination of RNA-mediated technologies may be a more efficient approach to gene inactivation, as supported by recent findings [76,87,300]. As it is established, when these technologies are combined with classical chemotherapy agents they can enhance the effectiveness of these drugs through silencing of drug resistance genes.

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