Synthetic Hammerhead Ribozymes as Therapeutic Tools to Control Disease Genes

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Abstract: Ribozymes are RNA molecules that have the ability to catalyse the cleavage and formation of covalent bonds in RNA strands at specific sites. The "hammerhead" motif, approximately 30-nucleotide long, is the smallest endonucleolytic *cis-acting* ribozyme structure found in natural circular RNAs of some plant viroids. Hammerhead ribozymes became appealing when it was shown that it is possible to produce *trans-acting* ribozymes directed against RNA sequences of interest.

Since then, gene-tailored ribozymes have been designed, produced and given to cells to knock down the expression of specific genes. At present, this technology has advanced so much that many hammerhead ribozymes are being used in clinical trials.

With this work we would provide some guidelines to design efficient *trans-acting* hammerhead ribozymes as well as review the recent results obtained with them as gene therapy tools.

Keywords: Hammerhead ribozymes, gene knockdown, gene therapy, gene drugs, synthetic oligonucleotides.

INTRODUCTION

RNA was traditionally viewed as a molecule that merely transfers genetic information from the nucleus to the cytoplasm or contributes to the structural demands of RNA-protein complexes. However, in 1982 the catalytic properties of RNA were discovered and profoundly changed this vision. For instance, t-RNA maturation by RNase-P and intron splicing are endonucleolytic reactions where the ribonucleic acid carries out the catalysis [Michel F. *et al.* (1989), Cech T.R. (1990), Pace N.R. and Brown J.W. (1995), Eckstein F. and Lilley D.M.J. (1996)]. Catalytic RNAs were named ribozymes because of their similarity to protein enzymes. Properties of ribozymes are strictly correlated with their conformational structures, often quite complex. The small sized catalytic RNAs of natural ribozymes from plant viroids and satellite RNAs of viruses are among the best characterised [Symons R.H. (1992), Bratty J. *et al.* (1993)]. The hammerhead catalytic molecule, approximately 30-nucleotide long, is the smallest endonucleolytic *cis-acting* ribozyme. It cuts its own sequence, arranged in multiple concatameres of the pathogenic RNA, to produce monomeric copies of the genome.

After the initial studies, it was shown that it is possible to produce *trans-acting* motives against any RNA of interest [Uhlenbeck O.C. (1987), Haseloff J. and Gerlach W.L. (1988)], immediately raising a great deal of interest. The structure-function relationship of the hammerhead catalytic molecule has been deeply investigated [Scott W.G. *et al.* (1995)] and numerous studies were published where gene-tailored, *trans-acting* ribozymes were successfully used in living cells, consistently knocking-down the expression of specific genes [Cotten M. and Birnstiel M.L. (1989), Eckstein F. and Bramlage B. (1999)]. This technology has become very popular in basic and applied research, especially in the fields of functional genomics and therapeutics. In fact, the inhibition of a single gene in a defined physiopathological context could clarify the gene contribution to the disease and the therapeutic potential of its inactivation.

Here we would like to provide some guidelines for a correct design of biologically active *trans-acting* hammerhead ribozymes and to review the most recent results obtained using them as gene therapy tools. Perspectives in ribozyme technology are also briefly indicated.

1.*TRANS-ACTING* HAMMERHEAD RIBOZYMES

1.1 The Structure of *trans-Acting* Hammerhead Ribozymes.

The hammerhead ribozyme consists of a conserved single-strand catalytic core (nucleotides indicated in Fig. **1**) at the junction of three Y-shaped, base-paired stems (Fig. **1**), [Scott W.G. *et al.* (1995)]. This *trans-acting* ribozyme is comprised of three distinct domains (Fig. **2**): a target-binding domain, a catalytic domain and a structural domain. Numbers are according to Hertel *et al.* (1992). The target-binding domain includes two far arms of a ribozyme sequence, able to pair with the target RNA to produce stems I and III. It represents the "antisense" moiety of a hammerhead ribozyme and confers the target specificity. The catalytic domain is a highly conserved, single stranded

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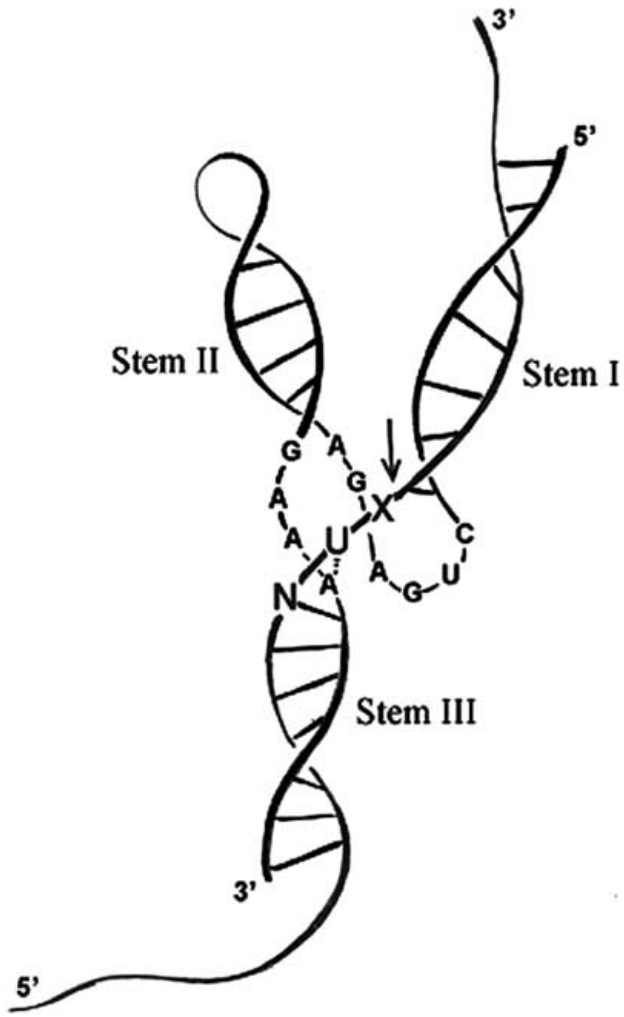

Fig. (1). Schematic view of the three-dimensional structure of a hammerhead ribozyme. Letters indicate the single stranded catalytic core consensus. The arrow marks the cleavage site down-stream the NUX consensus triplet. Stems I, II, III are indicated according to convention.

structure that connects stem I to stem II and stem II to stem III, respectively. This domain provides the specific geometry for the correct arrangement of magnesium ions and water molecules in correspondence of the scissile phosphate bond. Extensive analysis of the conserved catalytic domain was performed by systematic site-specific mutagenesis [Ruffner D.E. *et al.* (1990), Kuimelis R.G. and McLaughlin L.W. (1996)]. Results showed that any mutation produces a heavy reduction of the ribozyme cleavage rate. Stem II, the scaffolding domain, is necessary to maintain the geometry of the catalytic domain. Interestingly, the only requirement for the target substrate is a three-nucleotide consensus sequence at the cleavage site, NUX, where N = any nucleotide and X = A, C, U. As a consequence, it is possible to find plenty of cleavable triplets in all RNA targets. Systematic investigations of the relative cutting efficiency of different cleavable triplets indicated substantial differences among the various nucleotide combinations. Rough classifications of their reactivity in subsaturating or saturating conditions were published [Shimayama T. *et al.* (1995), Zoumadakis M. and Tabler M. (1995)].

The cleavage reaction catalysed by a hammerhead ribozyme consists in a trans-esterification of the phosphodiester bond that produces two fragments, the down-stream fragment containing a 5'hydroxyl and the up-stream one containing a 2',3'-cyclic phosphate [Uhlenbeck O.C. (1987)]. Divalent metal ions, usually Mg^{+2}, are essential for this ribozyme catalysis indicating that the ion not only assists in the correct RNA folding but also participates directly to the cleavage mechanism (Fig. **3)**, [Dahm S.C. and Uhlenbeck O.C. (1991)].

Kinetics of cleavage by hammerhead ribozymes was also thoroughly investigated. A Michaelis-Menten mechanism for the formation of ribozyme-substrate complex and the subsequent transition to products was found [Fedor M.J. and Uhlenbeck O.C. (1992), Hertel K.J. and Al. (1994)]. Experiments, performed under multiple turnover conditions on short synthetic substrates, were done mainly to compare the catalytic ability of different hammerhead sequences and to account for the effects of strength of binding on the overall cleavage kinetic [Fedor M.J. and Uhlenbeck O.C. (1992),]. We performed multiple turnover kinetic experiments to compare the cleavage activity of two ribozymes directed against different regions of the human O^6methylguanine-DNA methyltransferase (MGMT) mRNA [Citti L. *et al.* (1997)] and also to analyse the effects of chemical modifications on the catalytic activity of given ribozymes [Citti L. *et al.* (1999)]. As for long RNA substrates, the multiple turnover conditions cannot be applied because cleavage rates are generally several orders of magnitude lower than those measured for short substrates. Consequently, kinetic parameters were calculated under single turnover conditions, where varying excess amounts of the ribozyme are allowed to react with a constant concentration of substrate [Heidenreich O. and Eckstein F. (1992), Ellis J. and Rogers J. (1993), Hertel K.J. *et al.* (1994), Bertrand E.L. and Rossi J.J. (1994), Sioud M. (1997)]. The relative catalytic ability of different ribozymes can change depending on the relative length of the substrates. Usually, the long substrate is more informative for subsequent cell applications [Fedor M.J. and Uhlenbeck O.C. (1990)] but various factors can reduce the cutting efficiency of long substrates. In fact, long RNA molecules can fold in several ways [Birik K.R. *et al.* (1996)] and also are less mobile in solution.

Degrading enzymes, target-masking elements and compartment accessibility [Heidenreich O. *et al.* (1995)] can also affect the intracellular efficacy of ribozymes.

1.2 Designing *Trans-Acting* Hammerhead Ribozymes

Following the above observations, a defined set of steps must be taken when designing ribozymes:

a) Identification of NUX cleavage sites

b) Selection of the flanking sequences (annealing energies and sequence uniqueness)

c) Selection of accessible target sites within the RNA substrate

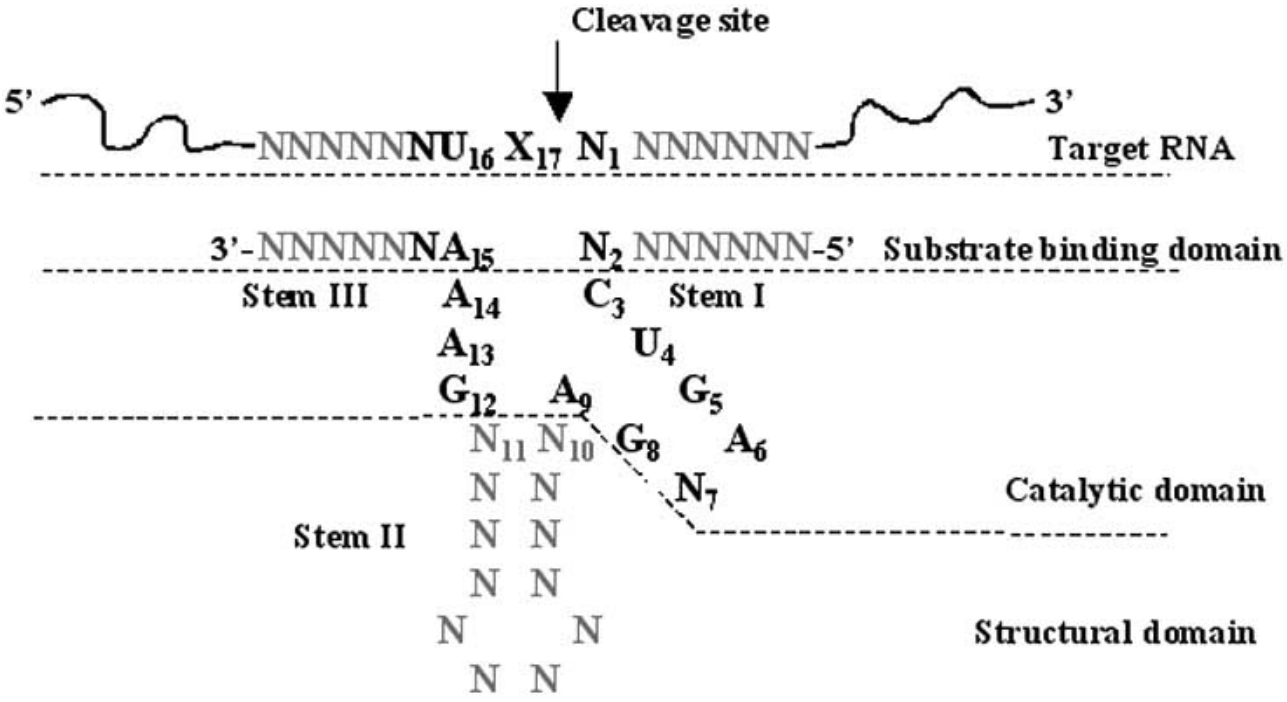

Fig. (2). Schematic representation of trans-acting hammerhead ribozyme. Dashed lines mark the different domains described in the text. Numbering accords to Hertel *et al.* (1992). The arrow indicates the scissile phosphate bond joining X_{17} to N_1 nucleotide.

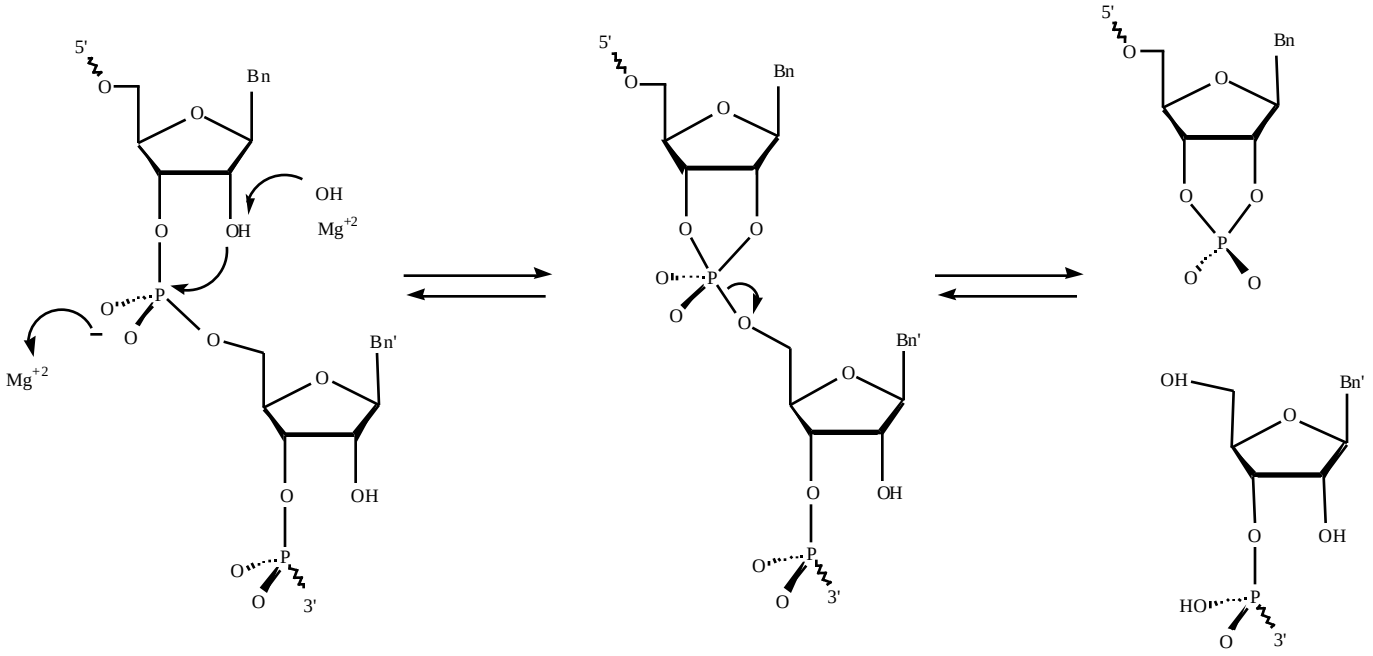

Fig. (3). Schematic representation of the cleavage reaction. A S_N2 nucleophylic substitution of the 2'OH on the adjacent phosphodiester bond produces a 2',3'-cyclic phosphate on up-stream fragment, leaving a 5'OH on the down-stream one [Slim G. an Gait M.J. (1991)]. The cooperative effects of Mg^{+2} are also indicated.

d) Verification of the suitability of ribozyme sequence to the structure-activity requirements.

Each point was shown to affect to some extent the catalytic efficiency of hammerhead ribozymes. In particular, a single major cause of ribozyme failure is arguably the inaccessibility of target sequence, when local stably annealed sequences are not available to pair with the ribozyme. RNA folding is a spontaneous process occurring both "*in vitro*" and "*in vivo*", depending on the thermodynamics of intramolecular pairing among complementary portions of a given RNA to produce secondary structures. A single RNA molecule may actually take on a number of different secondary structures with comparable free energy, as shown

by examples of the human *beacon* RNA **(**Fig. **4)**. A dynamic situation can be imagined, with all possible conformations interconverting each other, the most stable being the most represented. Whereas searching for cleavage sites and evaluating the pairing energies of flanking sequences (points a) and b)) can be done with common programmes for nucleic acids sequence management, the detection of RNA foldings is a more complicated task. Several methods for target selection have been developed, that can be classified as either experimental or predictive ("in silico"). A widely used experimental method measures the RNase-H sensitivity of substrate regions in the presence of short antisense oligonucleotides as proxy for annealing accessibility. It is assumed that cutting events map the accessible stretches of the RNA sequence [Scherr M. and Rossi J.J. (1998), Ho S.P. *et al*. (1998), Amarzguioui M. *et al.* (2000), Scherr M. *et al.* (2000)]. Similar methods were applied also to mixed computational/experimental studies to confirm computed previsions [Scherr M. et Al (2000)]. A more accurate method appeared in 1995 [Lieber A. and Strauss M. (1995)]: a library of ribozymes containing randomised binding domains was cloned and expressed in living cells enabling the selection of most efficient ribozymes. An alternative, successful experimental method was based on the "*in vitro*" selection of the best cutting sequences from a combinatorial collection of hammerhead ribozymes using the systematic evolution of ligands with the exponential enrichment (SELEX) method [Pan V.H. *et al.* (2001)].

As the experimental detection of accessible regions of RNA targets is difficult, expensive and time consuming, "in silico" predictive methods were developed based on computing technique introduced to predict RNA folding. They use a series of algorithms based on energy minimisation of thermodynamic parameters derived from continuously improving collections of experimental data produced with simple structural motives [Santa Lucia J.Jr. (1990), Jaeger J.A. and Al. (1989), Zucker M. (1989), Mathews D.H., Sabina J. *et al.* (1999)]. With these methods, several studies showed that the local secondary arrangement of RNA correlates with substrate accessibility and to the binding ability of oligonucleotides [Mathews D.H., Burkard M.E. *et al.* (1999), Amarzguioui M. *et al.* (2000), Scherr M. *et al.* (2000)]. Similar computational predictions were applied also for designing ribozyme [Denman R.B. (1993)].

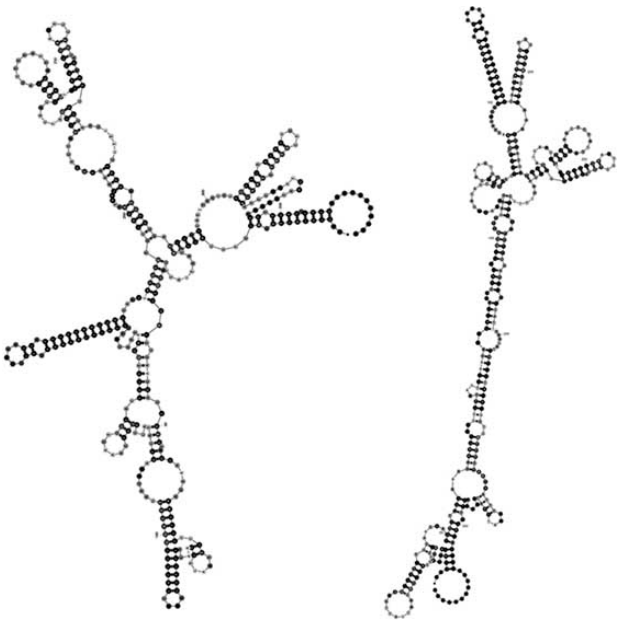

Fig. (4). Example of different secondary structures of an RNA sequence calculated with the M-Fold program. Both sequences although look quite different in fact display similar free energy of formation and are as likely to form.

We developed an integrated computing method to predict the most accessible sites on substrate RNA molecules for the selection of hammerhead ribozymes targets [Mercatanti A. *et al.* (2002)]. It consists of a series of successive steps: a) search of all NUX triplets occurring in the substrate sequence; b) selection of regions flanking each NUX triplets on the basis of imposed limits of pairing energy; c) computation of all optimal and suboptimal secondary structures for the entire RNA sequence; d) ranking of structures according to Boltzmann distribution and calculation of the "accessibility score" associated to each NUX target region, e) selection of possible targets showing the highest "accessibility score"; f) deduction of specific ribozyme sequence for each target and analysis of the most probable secondary geometry to verify its fulfilment of the structural requirements typical of a hammerhead ribozyme (Fig. **5**). This method enabled us to discard a considerable number of possible cleavage sites saving only those endowed with the most favourable conditions (Table **1**). Studies of the human bcl-2 mRNA that compare the actual biological inhibitory efficacy with predictions obtained with this method shows that the computing approach is highly prognostic [Mercatanti A. *et al.* (2002)]. The same method was applied to design hammerhead ribozymes against MGMT, survivin, *BUB-1*. All artificial ribozymes were highly active, producing significant gene inhibitions when given to the appropriate biological systems [Mariani L. *et al.* (2000), Pennati M., Colella G. *et al.* (2002), Pennati M. Binda M. *et al.* (2003), Musio A. *et al.* (2003)].

1.3 Shielding the Ribozymes from Degradation

The hammerhead ribozyme is the smallest catalytic RNA and it can be readily synthesised via automated chemical synthesis that facilitates biochemical and biophysical studies "*in vitro*". Unfortunately, such short RNA molecules display a very brief half-life in the biological environments. They are highly sensitive to ubiquitous ribonucleases, a feature that limited and delayed the use of ribozymes technology in all fields of basic and applied research. To overcome this serious limitation, two different approaches were developed, namely endogenous continuos synthesis *via* expression vectors and chemical protection of synthetic ribozymes.

The use of ribozyme expression vectors is a widely employed strategy to produce a permanent gene knockdown (see section of ribozyme applications). It is the obvious option for a gene therapy approach and displays all advantages of a persistent reduction of a target gene expression. It is suitable for the production of new phenotypes in long-term studies. Limitations underlying this

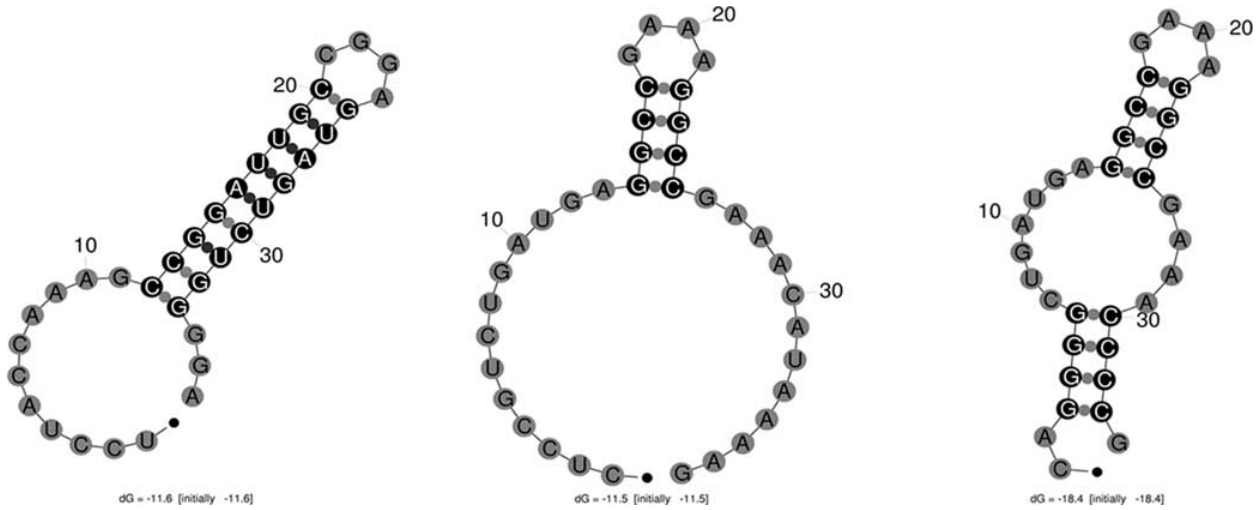

Fig. (5). Examples of self-structuring of three different trans-acting hammerhead ribozymes. A) Correctly folded ribozyme where stem II is the only annealed region. B) incorrect folding where the catalytic core is partially involved in one anomalous shifted pairing of stem II. C) incorrect pairing caused by the substrate binding arms that anneal with each other instead of binding the target.

Table 1. Selection of Target of Hammerhead Ribozymes According to the Computational Method Developed by Mercatanti *et al.* (2002)

Gene	GeneBank Accession number	Number of NUX cleavage sites	Selected Ribozymes
O^6methylguanineDNA methyl transferase, MGMT	M29971	89	6
Telomerase reverse trancriptase, hTERT	AF018167	385	8
Growth factor receptor, erbB2/neu	X03362	464	10
rad50	U63139	1001	62
survivin	NM_001168	271	13
Transcription factor, E2F1	M96577	285	13
Insulin-like growth factor receptor, IGF1-R	X04434	699	16
Glucokinase pancreatic splice variant, GCK	M90299	252	9
bcl2	M13994	916	43

All study is referred to human gene except *erbB2/neu*. The number of selected ribozymes ranges from 2 to 7 % of all possible NUX cleavage sites.

approach are: low transfection efficiency, a non-homogenous transcription level among various cells, a possible incorrect localisation of the ribozyme transcript, a frequent silencing of the trans-gene.

The second approach involves the use of chemically stabilised synthetic hammerhead ribozymes. This strategy is similar to conventional drugs administration, where a temporary pharmacological effect is obtained. The use of synthetic hammerhead ribozymes as "gene-drugs" has been successfully done in "*in vitro*" and "*in vivo*" experiments. There are some advantages in the use of short ribozymes: they are efficiently delivered to target cells (almost all cells are transfected) and accumulate intracellularly at considerable concentrations. Moreover, the transitory effect of a ribozyme administration could prove useful if a gene knockdown causes unwanted side-effects on tissues surrounding the target cells. The main problem posed by the use of synthetic hammerhead ribozymes relates to the chemical stabilisation of their short sequence. Many studies were done to produce synthetic molecules that were both highly catalytic and reasonably resistant to ribonucleases. A series of seminal observations produced with human and bovine serum showed that hammerhead ribozymes are degraded mainly by an RNaseA-like activity for which single stranded pyrimidines (primarily Uridine) are critical [Sproat B.S. (1996)], (serum is the model vehicle for "*in vivo*", systemic distribution of drugs and has high levels of nucleases). Consequently, chemical stabilisation strategies focused mainly on the phosphate and ribose moieties of single stranded portions of the ribozyme sequence. Also,

modified bases were tested but only few examples showed a better resistance to RNases [Biegelman L., Karpeisky A., Matulic-Adamic J. *et al.* (1995), Biegelman L., Karpeisky A., Usman N. (1995), Biegelman L., McSwiggen J. *et al.* (1995)]. The phosphate modifications (mainly phosphorothioates) [Stein C.A. (1988)], came in after a long experience using antisense oligonucleotides. Since phosphate groups contribute to three-dimensional structure of ribozyme [Ruffner D.E. and Uhlenbeck O.C. (1990), Shimayama T. *et al.* (1993)], phosphorothioates can be suitably introduced in the single strand elements of the target-binding domain, avoiding the catalytic core [Heidenreich O. *et al.* (1994)]. The most important modifications introduced to improve the nuclease resistance concern the sugar moiety. A lot of effective, stabilising sugar modifications (Fig. **6)**, that confer a better nuclease resistance to hammerhead ribozymes without affecting their catalytic activity, were extensively described [Sproat B.S. (1996), Biegelman L., Karpeisky A., Matulic-Adamic J. *et al.* (1995), Heidenreich O. *et al.* (1994)] and adopted in plenty of subsequent works.

Vehicles for efficient delivery are another factor that indirectly contributes to protecting ribozymes in different environments. Actually, viral particles as well as liposome-based or peptide-based vehicles substantially protect from nuclease demolition ribozyme-expressing vectors as well as short, synthetic, and catalytic molecules.

Another crucial issue affecting the ribozyme performance is represented by the efficiency of cell delivery, but we will not discuss this topic.

2. APPLICATIONS OF *TRANS-ACTING* HAMMERHEAD RIBOZYMES

During the last four years, approximately 100 papers were published that report a successful use of synthetic ribozymes as well as of ribozymes cloned in expression vectors. This paragraph will illustrate recent results concerning therapeutic developments.

The therapeutic use of hammerhead ribozymes depends on several factors that have to do with physiopathology, the histology of target tissues, the method and the efficiency of delivery. The main issues can be summarised as follows: a) the target gene must be crucial in causing/promoting the disease, so that its inhibition could likely induce cell death or revert the pathological phenotype; b) mRNAs must target the appropriate cell compartment; c) as many pathological cells as possible should be transfected with the active ribozyme or transduced by an appropriate ribozyme-expressing vector. All these criteria pose serious limitations to the use of ribozymes as therapeutic agents. This could explain why, despite encouraging "*in vitro*" and "*in vivo*" results, clinical experimentations are being seriously delayed.

2.1 Viral Infections

The use of ribozyme technology to counteract viral infection represents a theoretically canonical application of gene knockdown. In fact, only the infected cells expressing the viral genes would be targeted, leaving the cellular transcripts unaffected. The advantages of this new therapeutic strategy concern the possibility of targeting

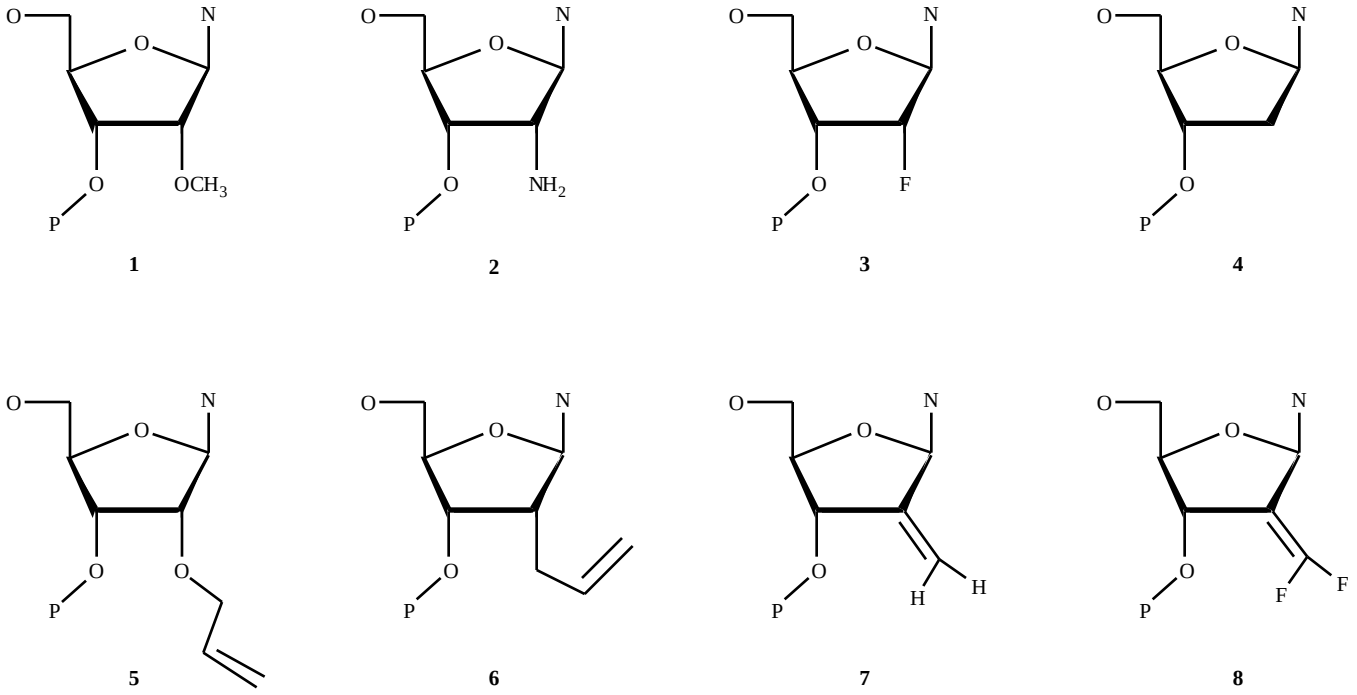

Fig. (6). Sugar chemical modifications that confer nuclease resistance to synthetic RNAs. 1) 2'-O methyl, 2) 2'-amino, 3) 2'-fluoro, 4) 2'deoxy, 5) 2'-O allyl, 6) 2'-allyl, 7) 2'-methylene, 8) 2'-difluoromethylene. Combinations of these modifications were used successfully in the cell administration of hammerhead ribozymes.

simultaneously a number of viral genes thereby overcoming the rapid mutation rate of some viruses. Many studies using ribozymes against viral infections addressed the influenza A virus [Lazarev V.N. *et al.* (1999)], hepatitis viruses HCV [Sakamoto N. *et al.* (1996), Ideo G. and Bellobuono A. (2002)], HBV [Feng Y. *et al.* (2001), Morrissey D.E. *et al.* (2002)], human cytomegalovirus HCMV [Trang P. *et al.* (2000)], herpes simplex virus HSV [Cantin E.M. *et al.* (1992), Trang P. *et al.* (2001)] and mainly the acquired immunodeficiency virus HIV. Targeting HIV with ribozymes could be effective in two stages of the viral life cycle: a) immediately after the infection prior to proviral DNA formation; and b) after the establishment of integrated provirus from which viral transcripts are produced. A stable expression of a 5'U5 leader targeted ribozyme protected T cell lines from HIV-1 infection [Yamada O. *et al.* (1994)]. Besides such protective therapeutic use of ribozymes, a lot of catalytic sequences acting against the expressed viral proteins from the integrated provirus were successfully studied. Ribozymes addressed to *tat*, *rev*, and *env* proteins of HIV produced an effective long-term protection against HIV-1 infection in a SCID-hu mouse model of *in vivo* thymopoiesis [Bai J. *et al.* (2002), Akkina R. *et al.* (2003)]. Antiviral therapeutic ribozymes have undergone clinical trials [Rossi J.J. (1999), Sullenger B.A. and Gilboa E. (2002)] Examples of synthetic ribozymes, which have undergone clinical trials, concern the anti-hepatitis C virus HEPTAZYME™ (Eli Lilly-Sirna Therapeutics), the anti-hepatitis B virus HepBzyme™ (Sirna Therapeutics) anti HIV-1 ribozyme OZ1 (Johnson & Johnson) or an anti-HIV multiple ribozyme gene (Ribozyome).

2.2 Neoplastic Pathologies

The use of ribozyme to control cancer growth is a quickly expanding area. A number of different target genes were investigated depending on the kind of tumors and the nature of cancer cells. It is hard to delineate an organic picture of all the ribozymes tested in this context, however, new therapeutics for cancer are being studied to hit different genes peculiar of the neoplastic diseases: oncogenes and cell proliferation genes, survival- and apoptosis-related genes, chimeric genes derived from chromosomal translocations, drug-resistance genes, angiogenic factors.

Actually, many oncogenes have been successfully targeted. In general, oncogenes belong to signal transduction pathways that control cell growth and proliferation and are frequently mutated and/or over-expressed in the malignant cell. The oncogene *ras* is an interesting target. The *Ras* family belongs to small GTPase enzymes. They are switches that coordinate extracellular stimuli with intracellular signalling pathways to activate appropriate biological responses. Interestingly, the oncogene *ras* shows a high frequency of single base mutations that can be recognised by specific ribozymes. Consequently, ribozymes directed against mutated codons of *ras* can selectively distinguish neoplastic from normal cells. Contributes concerning the reversal of malignant phenotypes by targeting point mutations of *H-ras* [Irie A. *et al.* (1999), Wang C.H. *et al.* (2002)], *K-ras* [Zhang Y.A. *et al.* (2000), Tokunaga T. *et al.* (2000), Kijima H. and Scanlon K.J. (2000)] and *N-ras* [Scherr M. *et al.* (1998)] were recently published.

Various tyrosine kinase receptors can participate to the malignant transformation due to over-expression or mutations that causes a sustained or altered signal transduction pathway. For instance, they induce anomalous cell proliferation or the establishment of an invasive phenotype. In this respect, successfully targeting the c-erbB-2/*neu* [Czubayko F. *et al.* (1997), Lui V.W. *et al.* (2001), Kiessling R. *et al.* (2002), Bi F. *et al.* (2001)], c-*met* [Abounader R. *et al.* (1999), Aboundaer R. *et al.* (2002), Jiang W.G. *et al.* (2001), Herynk M.H. *et al.* (2003)] and RET oncogenes were described [Santoro M. *et al.* (2002), Parthasarathy R. *et al.* (1999)].

Also, oncogenes belonging to the transcription factor family comprising *myc* or *myb* have been considered. Targeting such factors is quite complicated because they are normally involved in physiological processes such as proliferation, differentiation and apoptosis. C-*myc* is frequently over-expressed in tumors with poor prognosis. The ribozyme-mediated down-regulation of *myc* was recently shown to be very effective in tumors displaying high *myc* levels and high proliferating activity [Cheng J. *et al.* (2000)].

Very important targets for cancer therapy are all those gene specific of the malignant phenotype. Chromosomal rearrangements in cancer are frequent events that produce chimeric genes that are translated as fusion proteins. Fusion proteins are usually transforming and absolutely distinctive of the cancer cell. Chromosomal translocations present in leukaemia (e.g. the PML/RAR translocation in the acute promyelocytic leukaemia, the *bcr/abl* translocation in chronic myeloid leukaemia) give rise to chimeric mRNA species that are attractive targets for gene therapy as they are expressed exclusively in the tumor tissue. Ribozymes tailored to the junction region of the two mentioned chimeric RNAs produced in fact inhibition of growth in both types of leukaemia and represent promising therapeutic tools for bone marrow purging [Nason-Burkenal K., Allopenna J. *et al.* (1998), Nason-Burkenal K., Takle G. *et al.* (1998), Kubawara T. *et al.* (2001), Suwanai H. *et al.* (2002), Mendoza-Maldonado R. *et al.* (2002)].

Other targets were investigated concerning the cell survival and apoptosis mechanisms. Impaired apoptosis is a central step towards neoplasia, in that pro-survival proteins can promote tumourigenesis while pro-apoptotic act as tumor suppressors. Pro-survival factors as those belonging to Bcl-2 family are frequently over-expressed in tumors. Knocking-down their expression could represent a tool for a cytotoxic therapy. Bcl-2 was knocked-down with a ribozyme in different tumors. This treatment induces apoptosis and increases sensitivity of treated cells to apoptotic drugs [Dorai T. *et al.* (1997), Dorai T. *et al.* (1999), Gibson S.A. *et al.* (2000), Luzi E. *et al.* (2003), Poliseno L., *et al.* (2004)].

A similar approach was applied to survivin, another anti-apoptotic protein. This protein is over-expressed in most human tumors while is absent in normal adult tissues with only few exceptions at level of embryonic and foetal organs. Survivin is involved in the control of cell division and inhibition of apoptosis by inhibiting the caspases activation. With the method described above, we developed two ribozymes that caused considerable inhibition up to 90%, at

clonal level, of survivin in a metastatic melanoma cell line [Pennati M. *et al.* (2002)]. Such level of inhibition in the drug-refractory tumor resulted in a significant 7-fold increase of cis-platinum induced apoptotic cells suggesting a possible strategy to enhance the chemosensitivity of malignant melanoma. One of the previously described ribozymes was tested again in melanoma cells to account for its effect on γ-irradiation sensitivity [Pennati M. *et al.* (2003)]. A significant increase of radiation sensitivity was observed indicating for the first time the possibility of modulating the radiation sensitivity of human tumor cells by restoring their susceptibility to radiotherapy. Similar results were recently published on MCF-7 human breast cancer cells where apoptosis susceptibility was significantly increased after anti-survivin expression of hammerhead ribozymes [Choi K.S., *et al.* (2003)] and in human androgen-independent prostate cancer cells [Pennati M., *et al.* (2004)].

Telomerase is another enzymatic activity correlated to cell survival that probably plays an anti-apoptotic role through the replicative stabilisation of telomeres observed in many immortalised tumor cells. The telomerase RNA components (RNA template or mRNA sequence encoding the polymerase enzyme) represent potential good targets for developing antitumor strategies. However, conflicting results were reported [Folini M. *et al.* (2000)] and many questions (for example, in a number of cases the inhibition of telomerase doesn't produce telomere shortening, possibly because alternative pathways exist) need to be clarified before ribozymes can come to maturation as therapeutic tools [Ludwig A. *et al.* (2001), Folini M. *et al.* (2002)].

Drug resistance is a very important issue in cancer therapy and a number of different approaches based on ribozymes attempted to address it. The multidrug-resistance phenotype is frequently induced by cancer chemotherapy and represents a serious limitation to the current clinical treatments. One of the mechanisms of the multidrug resistance is the over-expression of p-glycoprotein (P-gp) that works as an efflux pump for various drugs. P-gp is encoded by a group of related genes termed MDR but only the MDR-1 gene is known to confer multidrug resistance and its over-expression is frequently found in resistant cancer cells. Targeting MDR-1 and associated proteins with specific ribozymes produces reversal of the resistant phenotype and represent a promising perspective for therapeutic applications [Kobayashi H. et Al (2001), Hatanaka H. *et al.* (2001), Nagata J. *et al.* (2002), Osada H. *et al.* (2003), Wang H. *et al.* (2003)].

However, drug resistance may also depend on cell factors different from MDR. For instance, the antiapoptotic/survival factors can adverse the effects of apoptotic drugs.

Another kind of resistance concerns the alkylating drugs whose pharmacological effects depends on the alkylation damage of DNA of target cells. DNA repair enzymes play a crucial role in this context. In particular, expression levels of the suicide enzyme O^6methylguanine-DNA methyl-transferase (MGMT) were linked to resistance of some cancer cells towards alkylating drugs such as alkylnitrosoureas or alkyltriazenes. We designed and tested synthetic hammerhead ribozymes against MGMT mRNA in mammalian cells expressing high levels of human MGMT. The use of synthetic short hammerhead molecules forced us to produce chemically stabilized ribozymes in order for them to survive in the cellular environment long enough to cover the half life of long-lasting MGMT protein [Citti L. *et al.* (1999)]. Transfection of hammerhead ribozyme increased the genotoxicity induced by alkyltriazene administration. Because the ribozyme acts directly on translation, it can inhibit only de-novo synthesis of the MGMT protein without affecting the long-lasting, pre-existing MGMT mRNA. A dual treatment with ribozyme and specific inhibitor of MGMT produced a dramatic increase of drug sensitivity [Mariani L. *et al.* (2000)] indicating the actual possibility to revert the drug-resistance phenotype in cancer treatment. Similar results were obtained in a model where ribozyme was stably transfected with an expression vector in target cells [Zhang Q. *et al.* (2001)].

Anti-angiogenetic therapy is another antiproliferative approach to combat cancer growth. Targeting vascular endothelial growth factor (VEGF) or related membrane receptors (Flt-1, KDR) with ribozyme proved to be a successful approach in model organisms [Ke L.D. *et al.* (1998), Parry T.J. *et al.* (1999)].

2.3 Cardiovascular Diseases

Cardiovascular diseases such as hypertensive vasculopathy, artery restenosis after angioplasty, vascular bypass graft occlusion and transplant coronary vasculopathy are yet another field where ribozymes were extensively applied in animal models. The best described mechanism of these vaso-occlusive processes rely on triggering the proliferation of vascular smooth muscle cells (VSCM) that invade the artery lumen.

Several factors, such as blood cells, humoral blood components (growth factors, cytokines, lymphokines, chemokines, hormones, glucose, dissolved gases…) or physical factors (pressure, sheer stress) were indicated as causative.

Two main targets were aimed to for counteracting the anomalous VSCM phenotypes with the ribozyme knockdown technique: a) cell proliferation and b) cell-induced mobility. The first aspect was developed mostly by down-regulating growth factors, signal transduction intermediates, cell cycle effectors, extracellular matrix remodelling. Among growth factors, the ribozyme knocking-down of platelet-derived growth factor (PDGF) A-chain efficiently inhibited the VSCM cell proliferation "*in vitro*" and the formation of neointima in an animal model [Hu W.Y. *et al.* (2001), Hu W.Y. *et al.* (2002), Kotani M. *et al.* (2003)]. Also, transforming growth factor-beta (TGF-β) was targeted with a hammerhead ribozyme. Those studies provide the evidence that selective cleavage of TGF-β mRNA produces a substantial inhibition of VSCM proliferation and neointimal formation "*in vitro*" and in animal models [Yamamoto K. *et al.* (2000), Teng J. *et al.* (2000), Su J.Z. *et al.* (2000)]. Other growth factors such as the vascular endothelial growth factor (VEGF) or related membrane receptors (Flt-1 and KDR) are not considered suitable targets because of their role in restoring the endothelial layer of artery vessels. However, because the microvessel formation is a late event of the neointima

development, targeting angiogenesis factors, including VEGF, has been recently suggested as a new strategy to inhibit restenosis [Fuchs S. *et al.* (2001)]. Many other proliferative factors were successfully targeted with ribozymes producing significantly consistent reduction of the VSMC proliferation. Among these, the *c-myb* transcription factor that is involved in the G_1/S transition after proliferative stimuli [Afroze T. and Husain M. (2001)]. Hammerhead ribozymes developed against *c*-myb mRNA inhibited VSCM cell proliferation "*in vitro*" and "*in vivo*" [Jarvis T.C., *et al.* (1996), Jarvis T.C. *et al.* (1996), Macejak D.G. *et al.* (1999)]. The anti-proliferative approach to counteract restenosis with ribozyme was used successfully "*in vitro*" against the transcription factor E2F-1 or cyclin E [Grassi G. *et al.* (2001)] and "*in vivo*" against the PCNA factor [Frimerman A. *et al.* (1999)]. Mechanisms regulating matrix accumulation and/or degradation were also investigated to inhibit the VSCM proliferative response to growth factors. Matrix metalloproteinase (MMP2) [Tsukioka K. *et al.* (2002)] and/or tenascin-C glycoprotein [Cowan K.N. *et al.* (2000)] were targeted to reduce restenosis and vascular remodelling. Similarly, the apolipoprotein-(a) [apo-(a)], an inactive plasminogen competitor, was successfully targeted in "*in vitro*" experiments to produce a block of VSCM growth [Morishita R. *et al.* (1998)]. Chemotaxis was also used to target leukocyte-type 12-lipoxygenase (12-LO) for down-regulating the production of arachidonic acid metabolites, involved in growth and chemotactic effects on VSCM cells. 12-LO targeting by locally delivered hammerhead ribozyme reduced significantly the neointima formation in an "*in vivo"* model of rat carotid balloon injury [Gu J.L., *et al.* (2001)].

3. CONCLUDING REMARKS

Despite their effectiveness in cultured cells, the use of ribozymes in clinical applications is very poor, thus stressing once again that "*in vitro"* and "*in vivo"* are deeply different approaches. In general, all oligonucleotide-based drugs (antisense, ribozymes, decoys, aptamers and more recently siRNAs) on the basis of their similar nature, share similar problems when applied "*in vivo*".

The internalization of electrically charged oligonucleotide molecules in cultured cells is generally inefficient, however there are several examples where gene "knock down" with oligonucleotides was achieved "*in vivo*" without any delivery system, these differences between "*in vitro*" and "*in vivo*" reflecting also different experimental conditions. For example, cultured cells are usually tested a few hours after the treatment while experiments in animal involve repeated or prolonged administrations to ensure a long exposure of the target tissues to oligonucleotides [Juliano R.I. *et al.* (1999)].

Free oligonucleotides, due to their hydrophilic properties, are quickly distributed to tissues and cleared through excretion thus living a short half-life in biological fluids. Excretion occurs primarily via the urinary tract and only in minor part through biliary excretion. To counteract this fast metabolic clearance, high single or repeated doses are necessary that can in turn induce toxicity. Oligonucleotides containing phosphorothioate are generally more toxic and induce more side effects than methylphosphonate or 2'Omethyl-modified oligomers [Agrawal S. and Zhao Q. (1998)].

Liver > kidneys > spleen > bone marrow are the main acceptors of therapeutic molecules [Sandberg J.A. *et al.* (1999), Agrawal S., *et al.*- (1997), DeLong R.K. *et al.* -(1997)]. In fact, tissue distribution is not homogenous and up to now this has considerably limited the clinical applications of antisense and ribozymes. However, recent progresses have made possible the clinical application of oligonucleotide-based drugs and the results of some clinical trials demonstrated that many critical issues affecting the "*in vivo*" administration of oligonucleotides and ribozymes were solved:

a) Systemic Administration and Toxicity

The various chemical modifications introduced in the synthesis of antisenses or ribozymes ensure resistance to nucleases, improve pharmacological properties and reduce toxicity [Henry S.P. *et al.* (1997), Wu H. *et al.* (1998), Usman N. and Blatt L.M. (2000), Kurreck J. (2003)]. Pharmacokinetics and tolerability in humans and monkeys of "ANGIOZYME®", an anti-cancer, anti-angiogenetic synthetic ribozyme that targets VEGF-R1 is paradigmatic in this respect [Sandberg J.A. *et al.* (1999), Sandberg J.A., Parker V.P. *et al.* (2000), Sandberg J.A., Sproul C.D. *et al.* (2000)].

b) Carriers to Improve Cell Uptake

Cell uptake was greatly improved with the introduction of suitable carriers for systemic administration, in addition to the simple injection or infusion methods reported in animal experiments [Sandberg J.A. *et al.* (1999), Pavco P.A. *et al.* (2000)]. Carriers improve both membrane binding and cell internalisation and also protect against biochemical agents such as the ubiquitous nucleases. Cationic lipoplexes are an example of successful carriers. Unfortunately, most of the commercially available cationic lipids are unstable in serum therefore most of "*in vivo*" administrations use alternative tools such as polymeric matrices [Aigner A. *et al.* (2002)], dendrimers [Yoo H., Juliano R.L., (2000)], nanospheres and biodegradable polymers [Putney S.D. *et al.* (1999), Jackson *et al.* (2002)], receptor ligands or antibodies [Biessen E. *et al.* (1999)] and peptidic carriers [Morris M.C. et Al (1997), Schwarze S.R., Dowdy S.F. (2000)]. Successful applications are reviewed elsewhere [Hughes M.D. *et al.* (2001), Pouton C.W., Seymour L.W. (2001)].

c) Cell- and Tissue-Specific Delivery

The heterogeneous distribution of oligonucleotides after systemic administration depends on different accessibility and/or ability on the part of specific cells to internalize the synthetic molecules. Consequently, several approaches were designed to improve targeting.

i Receptor-Mediated Endocytosis

Some strategies are based on the use of receptors. Examples are: the transferrin receptor [Hudson A.J. *et al.* (1999)], the mannose receptor [Rojanasakul Y. *et al.* (1997)],

the folate receptor [Li S. *et al.* (1998)] and the asialoylglycoprotein receptor [Biessen E.A.L. *et al.* (1999)].

ii Local Delivery

Oligonucleotide drugs combined with suitable carriers were successfully given locally to solid tumors [Schawab G., *et al.* (1994)], in brain [Khan *et al.* (2000)] or at site of stent-induced stenosis [Frimerman *et al.* (1999)].

iii Ex-Vivo Administration and Re-Infusion

An example is the autologous implant of haematopoietic progenitor cells transduced with the anti-HIV ribozyme, OZ1, now under phase II clinical trial (Johnson & Johnson Research). This cell-specific targeting can be applied when stable expression of the therapeutic gene is required.

d) Allosteric Ribozymes (Cell Specific-Ribozymes)

This approach is based on site-activable ribozymes at the target cells. Allosteric ribozymes combine the "aptamer" and the ribozyme characteristics to give a molecular tool with allosteric properties. "Aptamers" are short nucleic acid molecules, obtained after "*in vitro*" selection of combinatorial libraries of random sequences, whose three-dimensional structure fits the molecular geometry of a given selecting agent. That is, they are able to bind, like antibodies, to specific ligands such as proteins, other nucleic acids or little molecules such as drugs [Ellington A.D. and Szostak J.W. (1990), Tuerk C. *et al.* (1993)]. This "*in vitro*" selection method, applied to combinatorial ribozyme sequences, would produce allosteric ribozymes whose catalytic cleavage activity is turned-on only when a specific selected ligand binds to the "aptamer" moiety of the molecule. An allosteric ribozyme is designed to interact specifically with molecules peculiar of a given pathological phenotype and therefore should obtain cell-specific therapeutic effects. In this way, many limitations imposed by "*in vivo*" ribozyme-delivery could be circumvented and that would allow a decisive acceleration of ribozymes therapeutic applications into clinical settings.

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