Catalytic nucleic acid enzymes for the study and development of therapies in the central nervous system

Review Article

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Abbreviations: β-site APP cleaving enzyme, (BACE); 7-amino actinomycin D, (7AAD); Aβ-amylloid peptide, (Aβ); Alzheimer’s disease, (AD); amyloid precursor protein, (APP); anaplastic lymphocyte kinase, (ALK); arabis mosaic virus, (sArMV); bearing intracranial BT; blood-brain-barrier, (BBB); central nervous system, (CNS); chicory yellow mottle virus type 1, (sCYMV1); Deoxynucleotide enzymes, (DNAzymes); dihydrolipoamide succinyltransferase, (DLST); epidermal growth factor receptor, (EGFR); external guide sequences, (EGS); Fas ligand, (Fas-L); Herpes Simplex Viral, (HSV); Interleukin-10, (IL-10); mitochondrial respiration generator of truncated DLST, (MIRTD); phosphoinositol-3-phosphate kinase, (PI3PK); proliferating cell nuclear antigen, (PCNA); Proliferative vitreoretinopathy, (PVR); protein kinase Cα, (PKCα); recombinant adenovirus associated viral, (rAAV); retinal detachment, (RD); Retinitis pigmentosum, (RP); RNA dependent protein kinase, (PKR); satellite RNA of tobacco ringspot virus, (-sTRV); scatter factor/hepatocyte growth factor, (SF/HGF); scrambled ribozyme, (sRz); untranslated regions, (UTR); Varkud Satellite, (VS); vascular endothelial growth factor, (VEGF)

Summary

Nucleic acid enzymes have been used with great success for studying natural processes in the central nervous system (CNS). We first provide information on the structural and enzymatic differences of various ribozymes and DNAzymes. We then discuss how they have been used to explore new therapeutic approaches for treating diseases of the CNS. They have been tested in various systems modeling retinitis pigmentosum, proliferative vitreoretinopathy, Alzheimer's disease, and malignant brain tumors. For these models, effective targets for nucleic acid enzymes have been readily identified and the rules for selecting cleavage sites have been well established. The bulk of studies, including those from our laboratory, have emphasized their use for gliomas. With the availability of multiple excellent animal models to test glioma treatments, good progress has been made in the initial testing of nucleic acid enzymes for brain tumor therapy. However, opportunities still exist to significantly improve the delivery and efficacy of ribozymes to achieve effective treatment. The future holds significant potential for the molecular targeting and therapy of eye diseases, neurodegenerative disorders, and brain tumors with these unique treatment agents.

I. Introduction

Ribozymes are RNA molecules that act as enzymes in the complete absence of protein. The first examples of such catalysts discovered in the early 1980s were the self-splicing group I intron from Tetrahymena thermophila and the RNA moiety from Ribonuclease P (RNase P) (Kruger et al, 1982; Guerrier-Takada et al, 1983). Since their initial discovery, RNA catalysts have been found in the self-splicing group II introns, plant virusoid and viroid RNAs, hepatitis delta virus satellite RNA, a transcript from the mitochondria of Neurospora crassa, and ribosomal RNA (Buzayan et al, 1986; Forster and Symons, 1987; Wu et al, 1989; Saville and Collins, 1990; Bonen and Vogel, 2001; Steitz and Moore, 2003).

Most of the naturally-occurring ribozymes catalyze intramolecular reactions in their native context, except for RNase P and ribosomes. The catalytic centers of these naturally self-cleaving ribozymes have been identified and engineered as agents to catalyze intermolecular reactions, that is, ribozymes that cleave heterologous RNA molecules (Haseloff and Gerlach, 1988; Hampel and Tritz, 1989). Substrate recognition for most of the known...
ribozymes is mediated by Watson-Crick base pairing, whereas, substrate binding for RNAses P and ribosomes involve topological properties of the substrate (Yuan and Altman, 1995; Frank et al, 2000; Nissen et al, 2000). Since the ribozyme sequences involved in substrate binding are not involved in the chemical step of catalysis, the specificity of ribozymes can easily be changed to target virtually any RNA of interest. Also, ribozymes only require a neutral pH and divalent cations to catalyze the cleavage of the substrate RNA. These properties make ribozymes highly desirable as agents for inhibiting gene expression at the mRNA level.

More recently, small sequences of DNA capable of site specific cleavage of RNA targets have been developed through in vitro evolution (Santoro and Joyce, 1998). The most commonly used 10-20 enzymes have catalytic properties reminiscent of the plant virusoid and viroid self-cleaving RNAs (Santoro and Joyce, 1998).

Ribozymes and catalytic DNAs have been exploited for use as inhibitors of gene function in many different cellular systems and delivered to targets by different modalities. A number of reviews have been written that describe these applications (Lewin and Hauswirth, 2001; Wright and Kearney, 2001; Emilsson and Breaker, 2002; Puerta-Fernandez et al, 2003; Scherer and Rossi, 2003; Trulzsch and Wood, 2004). The focus of this review is to describe how nucleic acid enzymes have been employed to study and potentially treat diseases of the CNS. We will summarize the different disease indications, highlighting the targets that were chosen and the outcome. Consideration will be given to the advantages and disadvantages of expressing the ribozyme or using a synthetic nucleic acid enzyme. We will include a discussion of delivery of nucleic acid enzymes by local or systemic administration taking into consideration, blood-brain-barrier (BBB) issues, status of the CNS as an immune-privileged site, stability of the ribozymes once delivered, along with use of appropriate animal models for studying and treating malignant gliomas.

II. Small catalytic RNAs

Small ribozymes typically have catalytic motifs that range from 30 - 150 nucleotides in length. The motifs were originally isolated from autocatalytic RNAs found in plant virusoid and viroid RNAs, hepatitis delta virus, and the Varkud Satellite (VS) RNA from Neurospora. These ribozymes appear to function naturally in the replicative cycles of the selfish RNA molecules. Ribozymes typically catalyze an intramolecular trans-esterification reaction leaving a 5'-OH and a 2', 3'- cyclic phosphate as termini at the site of cleavage. In all cases, the small ribozymes have been engineered to cleave heterologous RNAs in a trans-reaction (Haseloff and Gerlach, 1988; Hampel and Tritz, 1989; Branch and Robertson, 1991; Guo and Collins, 1995). The targeting and binding of the heterologous substrates is mediated by Watson-Crick base pairing. In this review we will focus on the hammerhead and hairpin ribozymes because, as of this writing, there were no examples of the hepatitis delta virus or VS ribozymes being used for studies in the CNS.

A. Hammerhead ribozyme

The hammerhead ribozyme is a two-dimensional catalytic motif found primarily associated with plant virusoid and viroid RNAs, although there are three examples of hammerhead ribozymes isolated from satellite RNAs in animals (Forster et al, 1988; Ferbeyre et al, 1998; Rojas et al, 2000). The secondary structure of the hammerhead ribozyme, shown in Figure 1A, is a conserved core of 10 single-stranded nucleotides surrounded by three base-paired helices, also known as stems. Stems 1 and 3 are formed by the base pairing between the ribozyme and its target. Whereas, stem - loop 2 has a 4 base-pair helix and an unpaired tetraloop and is ultimately dispensable for catalytic activity (Hendry et al, 1995). The base pairing in stems 1 and 3 is unrestricted with respect to sequence, and cleavage can occur after any NHH (N=any nucleotide; H = A, C or U) tri-nucleotide sequence in the target RNA (Kore et al, 1998). This gives the hammerhead ribozyme a very large pool of potential cleavage sites in any RNA of interest. The hammerhead ribozyme has also been altered by ancillary RNA motifs appended to its termini that can modify or augment the activity of the ribozyme in the cellular environment (Warashina et al, 2001; Kawasaki et al, 2002). The small size of the hammerhead ribozymes and its simple targeting rules have made it the most studied and utilized catalytic RNA for down-regulating gene activity in vitro and in vivo (Puerta-Fernandez et al, 2003).

B. Hairpin ribozyme

The hairpin ribozyme was first isolated as the catalytic center of the negative strand of the satellite RNA of tobacco ringspot virus (-sTRV). Subsequently, hairpin catalytic motifs were isolated from the satellite RNAs of chicory yellow mottle virus type 1 (sCYMV1) and arabis mosaic virus (sArMV) (Kaper et al, 1988; Rubino et al, 1990; Hampel, 1998). The secondary structure of the hairpin ribozyme is given in Figure 1B. The hairpin ribozyme isolated from -sTRV can be designed to cleave at any BNGUC (B=G, C, U; N= any nucleotide) sequence in the target RNA of interest (Hampel, 1998; Shippy et al, 1999). Initially, the ribozymes from sCYMV1 and sArMV were found to be non-catalytic. Subsequently, these ribozymes were engineered to be catalytic and designed to cleave any target RNA at any NGUA sequence present in the target RNA (DeYoung et al, 1995). Further refinements to the targeting rules for the hairpin ribozyme were developed to optimize the cleavage reaction. Unfortunately, the enhancements that increased the catalytic efficiency of the hairpin ribozyme were usually specific to the cleavage site chosen. Therefore, optimizing the hairpin ribozyme for a particular cleavage site required empirical testing, the description of which is beyond the scope of this review. Several excellent reviews have been written that discuss these modifications and the protocols necessary to optimize the hairpin ribozyme (Hampel, 1998; Shippy et al, 1999).
III. Ribonuclease P

RNase P is found in organisms throughout nature and processes precursor tRNAs, releasing 5’ sequences and mature tRNAs. Studies of the substrate requirements for human and bacterial enzymes show that the minimal substrate for either enzyme resembles a segment of a tRNA molecule. This structure can be mimicked by uniquely designed antisense RNAs that pair with the target RNA. These antisense molecules are termed external guide sequences (EGS), and when bound to the target RNA, resemble the 5’ terminus of immature tRNA molecules that can then serve as substrates for RNase P (Forster and Altman, 1990; Altman, 1995). One advantage of this system is that the cellular environment is optimal for RNase P catalysis and theoretically should promote the maximum efficiency for the ribozyme.

RNase P has been used to regulate the expression of NMDA receptors in primary rat neurons. The EGS was designed to affect a site that is present and accessible in the mRNAs of all NR1 splice variants (Yen et al, 2001). Expression of the EGS from Herpes Simplex Viral (HSV) vectors led to the reduction of functional cell surface NMDA receptors as determined by the neuro-protective action of the EGS in an excitatory assay in primary astrocyte cultures. The expression cassette for the EGS had an interesting design. It was flanked 5’ and 3’ by autocatalytic hammerhead modules (Figure 1C), which led to the excision of the EGS with precise 5’ and 3’ termini. This also allowed for the synthesis of a precise transcript from a Pol II expression cassette. The use of RNase P for these types of studies is not limited to targeting ubiquitous sites. One could also engineer the EGS to specifically bind to any of the NR1 splice variants and down-regulate their expression.

These results demonstrate the potential utility of the EGS and RNase P for studying the role of NMDA receptors in neural development and neurotoxicity. This preliminary study (Yen et al, 2001) to assess the ability of RNase P to knockdown NR1 easily could be expanded to in vivo animal studies using the HSV vectors developed to express the EGS.

IV. Deoxynucleotide enzymes

Deoxynucleotide enzymes (DNAzymes) are small DNAs that are capable of catalyzing several different types of reactions (Li and Breaker, 1999; Cairns et al, 2002). Most of the DNAzymes promote the cleavage of RNA molecules. To date, no naturally-occurring DNAzymes have been found in nature, but they can be designed in the laboratory to perform specific biochemical functions. DNAzymes are typically small, usually containing only a few nucleotides, and are designed to fold into specific tertiary structures that can catalyze particular chemical reactions. In the case of RNA cleavage, DNAzymes can be designed to target specific RNA sequences and introduce a nick or cut into the RNA molecule.

Figure 1. Canonical secondary structures for the ribozymes used to study and treat various eye and CNS diseases as presented in this review: (A) hammerhead ribozyme, (B) hairpin ribozyme, (C) RNase P RNA subunit, (D) 10-23 DNAzyme. Arrows denote sites of cleavage in the substrate RNA. N = any nucleotide; R = purine; Y = pyrimidine; and H = A, C or T. Structures obtained from: (A and B, McKay and Wedekind, 1999), (C, Lilley, 2000), (D, Emilsson and Breaker, 2002).
DNAzymes have been isolated. These catalysts are usually produced by in vitro evolution and the 10-23 DNAzyme (Figure 1D) has been the most studied for knockdown of mRNA. The RNA cleaving DNAzymes have very similar catalytic properties to ribozymes isolated from plant virusoids and viroids. They produce 5'-OH and 2',3'-cyclic phosphate termini at the cleavage site and targeting is determined by Watson-Crick base pairing (Joyce, 2001). The 10-23 DNAzyme and its derivatives have been used by a number of investigators to downregulate gene expression in vitro and in vivo (Emilsson and Breaker, 2002).

DNAzymes have been used to understand the role of laminin in axon regeneration. DNAzymes were designed against the γ 1 chain of laminin and introduced into the mossy fiber hippocampal slice culture model. The treatment with the DNAzymes inhibited mossy fiber regrowth and indicated a critical role for laminin in this process (Grimpe et al, 2002). The investigators studied this process further by using DNAzymes to inhibit xylosyl-transferase-1 (Grimpe and Silver, 2004). Down-regulation of this enzyme showed how the presence of glycosaminoglycan side chains on chondroitin sulfate proteoglycans, a component of the extracellular matrix, prevented regeneration after spinal cord injury. These studies made excellent use of organotypic tissue models for neuronal regeneration. However, further experimentation will be needed in animal models to determine whether inhibiting these genes significantly aids in neural regeneration.

V. Diseases of the eye
A. Retinitis pigmentosum
Retinitis pigmentosum (RP) is characterized by the loss of rod photoreceptor function and viability with 50% of the cases being sporadic and the remainder showing X-linked hereditary patterns (O'Neill et al, 2000; Farrar et al, 2002). RP is associated with 150 different mutations in the rhodopsin and peripherin genes. A further 24 loci have been mapped for RP but the affected genes remain to be isolated (Farrar et al, 2002).

RP is a dominant disorder with a large allelic genetic heterogeneity. This disease characteristic provides a unique opportunity to utilize ribozymes for treatment of this disease. One approach could involve targeting of a conserved site in the 3’ untranslated regions (UTR) of the allelic variants. Cleavage in the UTRs would result in the downregulation of both the wild-type and mutant alleles. Replacement genes engineered with modified UTRs that cannot be cleaved by the ribozymes could be introduced and expressed in the ribozyme-treated cells to produce functional proteins. An alternative approach could involve targeting the ribozyme against the known genetic polymorphisms found in RP genes. This would allow for the re-introduction and expression of the wild-type gene that could not be cleaved by the ribozyme (Millington-Ward et al, 1997; Drenser et al, 1998).

Hammerhead ribozymes for both of these approaches have been developed and characterized by in vitro cleavage assays, as well as, in the P23H (histidine substituted for proline at position 23) transgenic rat model (LaVail et al, 2000). This model represents one of the most common polymorphisms identified in human RP. Ribozymes introduced into this model by recombinant adenovirus associated viral (rAAV) vectors were able to slow the progression of photoreceptor degeneration compared to control animals. The results from these experiments illustrate the potential effectiveness of using ribozymes to treat dominant allelic disorders of the CNS. Again, more extensive studies in animal models would be required to take ribozymes into the clinic to treat RP.

B. Proliferative vitreoretinopathy
Proliferative vitreoretinopathy (PVR) is the principal reason why retinal repair surgery fails in roughly 5%-20% of patients with retinal detachment (RD) (Frenzel et al, 1998). Two of the hallmarks of PVR are the migration and proliferation of a variety of cells including fibroblasts, glia, and retinal pigment epithelial cells. The aberrant cell proliferation leads to the formation of neomembranes and ultimately causes the retina to contract and re-detach.

Our laboratory, in collaboration with Immusol, Inc. (San Diego, CA), has investigated use of a DNA-RNA chimeric hammerhead ribozyme (Taylor et al, 1992) targeting proliferating cell nuclear antigen (PCNA) in the dispase-induced rabbit PVR model (Mandava et al, 2002). PCNA is a critical cell cycle controlling factor in a variety of cell types, therefore, the ribozyme to PCNA has the ability to target and affect all cell types involved in disease progression. The ribozyme, the structure of which includes RNA and DNA components as shown in Figure 2, was developed to target a site in the rabbit PCNA gene that was conserved in sequence to the human gene. Ribozymes administered into the vitreous were stable. The ribozyme was uncleaved in the vitreous up to 240 min after injection, whereas, the half-life was less than 1 min in serum. Intravitreal injection of the ribozyme into rabbit eyes previously administered dispase to induce PVR prevented the development of, or slowed progression of the disease. Sham-treated rabbits, or those given scrambled ribozyme (different targeting sequence), developed severe PVR ending in retinal detachment. Based on the data a multi-institutional clinical trial was initiated to treat PVR in humans using the PCNA-targeted chimeric ribozyme. As well, the results from this study helped to validate a newly-developed animal model for PVR. The features of a cell cycle targeted ribozyme also made it an attractive candidate for treatment of brain tumors. Later, our work with the PCNA-targeted ribozyme in vitro with glioma cell models and in vivo with a rat brain tumor model will be presented.

VI. Diseases of the brain
A. Alzheimer’s disease
Alzheimer’s disease (AD) is a neurodegenerative disorder involving the deposition of senile plaques in the brain. The plaques consist of aggregates of a 4 kDa β-amyloid peptide (Aβ). The peptide is produced by proteolytic processing of the amyloid precursor protein (APP) by β- and γ-secretases at the N- and C-termini,
respectively (Li, 2001). An accumulation of the cleavage products, 39-43 amino acid β-amyloid peptides, are the major cause of pathogenesis in AD according to the amyloid hypothesis (Hardy, 2002).

β-secretase or β-site APP cleaving enzyme (BACE) makes an attractive target for intervention in the progression of AD. Recently, a hammerhead ribozyme against the BACE gene was designed and appended with a constitutive transport element on its 3’ terminus. The ribozyme genes were transfected into HEK293 cells and expression was driven by the tRNAval promoter (Nawrot et al, 2003). Maximal ribozyme expression was seen after 24 hr post transfection that resulted in a 95% reduction of the BACE mRNA relative to the lipid-treated control cells. The decrease in the mRNA was followed by a 90% reduction in the biosynthesis of BACE. Ultimately, the decreases in BACE mRNA and protein levels led to a decrease in the detection of Aβ in cell lysates and culture medium.

In two other studies, ribozymes were employed to determine the functional relevance of other genes implicated in AD. One study was able to assign a function to the mitochondrial respiration generator of truncated DLST (MIRTD) gene; this newly-discovered gene was actually a truncated version of dihydrolipoamide succinyltransferase (DLST) (Kanamori et al, 2003). The MIRTD gene is absent in 50% of AD patients, providing a possible correlation of a DLST genotype with AD. A dimeric hammerhead ribozyme (maxizyme) was targeted against the MIRTD gene. SH-SY5Y neuroblastoma cells transfected with both the maxizyme-left and maxizyme-right constructs had reduced MIRTD expression compared to SH-SY5Y cells transfected with maxizyme-left or maxizyme-right constructs only. The ribozyme expressing cells had a greater sensitivity to hydrogen peroxide and a significantly reduced rate of respiration, implicating MIRTD in the assembly of the cytochrome C complex.

Data from another AD study implicated a double-stranded RNA dependent protein kinase (PKR) in disease progression (Onuki et al, 2004). A library of hammerhead ribozyme genes was employed to identify genes involved in tunicamycin-induced apoptosis of SK-N-SH cells. Results from screening the ribozyme library led to the identification of PKR, which subsequently was found to be elevated in the brains of AD patients.

The experiments on BACE are preliminary and the results need to be repeated in neuronal cells and ultimately in animal models to determine if the knock-down of BACE will be a potential therapy for AD. The work on MIRTD may not lead directly to a therapeutic agent but may provide a marker or predictor of AD pathogenesis. The elevated phosphorylated PKR seen in SK-N-MH neuroblastoma cells was also found in the brains of AD patients. If these conditions can be reproduced in an animal model of AD, ribozyme targeting of this gene can enter preclinical development and potentially result in a drug for treatment for AD.

B. Malignant brain tumors

Glial neoplasms are the most common of primary malignant tumors of the central nervous system (CBTRUS, 2002). The prognosis for glioma patients is discouraging (McCarthy et al, 2002). They are a significant cause of death in young adults and in children (Pendergrass et al, 1987). The dismal outlook for most brain tumor patients is due to the inability of conventional therapies (surgery, radiation, and chemotherapy) to completely eliminate all glioma cells. Several factors contribute to the inefficacy of these treatments including the inability to ressect the tumor cells based upon their precarious locations within the brain, the heterogeneity of the tumor cell population, and the infiltrative nature of the glioma cells. There is a necessity to explore alternative adjunctive therapies that target brain tumor cells, since the
regeneration of damaged neurons within the brain is quite limited. Any damage sustained to them during treatment is likely permanent and their loss may have detrimental effects on patient quality of life.

1. Hairpin ribozyme to downregulate mutant epidermal growth factor receptor

One such alternative treatment was the development of the hairpin ribozyme to downregulate the expression of the mutant epidermal growth factor receptor (EGFR), termed EGFRvIII, in glioblastoma cells. This is a common mutation of the EGFR gene present in as many as 50% of all glioblastomas (Sugawa et al, 1990; Ekstrand et al, 1994; Moscatello et al, 1997; Wikstrand et al, 1997). The EGFRvIII mutation is an 801bp deletion of exons 2 to 7 of the EGFR gene. Themutation is associated with the amplification of EGFR in glioblastoma multiforme and other tumor types (Garcia de Palazzo et al, 1993; Moscatello et al, 1995) and has led to the use ofthis marker as a prognostic indicator (Huncharek and Kupelnick, 2000; Zhou et al, 2003) as well as a target for antibody therapy (Kuan et al, 2001). The EGFRvIII mutation includes a GGUA sequence that lies just downstream of the fusion junction of exon 1 with exon 8. Three versions of the hairpin ribozyme were tested and one, the sCYMV1 ribozyme, was chosen for further study based on cleavage assays (Halatsch et al, 2000). When the sCYMV1 ribozyme was transduced into EGFR-overexpressing U87MG-ΔEGFR glioma cells, the ribozyme inhibited proliferation by 69%. However, clonogenicity assays in soft agar demonstrated that anchorage independent growth was inhibited at greater than 95% compared to mock transected or U87MG-ΔEGFR cells transfected with empty vector or disabled ribozyme. These data indicate that the ΔEGFR may affect the interaction of glioma cells with extracellular matrix proteins, instead of affecting cell proliferation directly. This study, besides further implicating this genetic change as a significant event in the malignant development of glioma cells, also suggests that this gene would be a good target for developing a ribozyme therapeutic.

2. Hammerhead ribozyme that targets anaplastic lymphocyte kinase

Hammerhead ribozymes have been used to study the role of anaplastic lymphocyte kinase (ALK) in promoting the growth of malignant glioma cells. A panel of glioma cell populations, selected for stable expression of ribozymes targeting ALK, included cell populations with low, medium and high residual ALK expression. The glioma cell populations were then implanted as xenografts into nude mice to assess the affects of variable ALK expression levels on signaling and tumor growth (Powers et al, 2002). Tumor growth and pleiotropin signaling by way of ALK in the ribozyme-expressing xenografts was reduced in a gene dose-dependent manner corresponding to the low, medium, and high residual ALK expression of the implanted cell populations. The authors showed that the pleiotropin-ALK signaling pathway was essential for the growth of the glioma cell line U87MG in vivo, since reduced signaling resulted in growth inhibition of glioma tumors in vivo. Their data suggested that drugs developed to inhibit this pathway could be efficacious in the treatment of glioma. An acceptable treatment for brain tumors might involve a drug that slows proliferation of tumor cells. This would allow the patient better quality survival time, whereas treatments associated with induction of tumor cell necrosis may involve inflammatory components resulting in lesser quality of life (Dewey et al, 1999; Cowsill et al, 2000).

3. Ribozyme to target scatter factor/hepatocyte growth factor and c-met

In an elegant series of experiments a hammerhead ribozyme was used to target the multifunctional growth factor, scatter factor/hepatocyte growth factor (SF/HGF), and its receptor c-met. The ribozyme was expressed in a U1-ribozyme expression cassette which imparts greater stability to the ribozyme as well as compartmentalizing the ribozyme to the nucleus (Abounader et al, 1999). Initially, glioma cells either transfected or transduced with the U1-ribozyme gene exhibited reduced tumorigenicity in a xenograft mouse model compared to control cells transfected or transduced with empty vector. Subsequent findings showed that c-met activation by SF/HGF inhibited apoptosis by activating the phosphoinositol-3-phosphate kinase (PI3PK)-dependent and Akt (protein kinase B)-dependent pathways (Bowers et al, 2000). Inhibition of the SF/HGF c-met autocrine loop by U1 expressed ribozymes also prevented angiogenesis and promoted apoptosis in a nude mouse xenograft model compared to controls that only expressed the U1 snRNA (Abounader et al, 2002). The development of ribozymes targeting SF/HGF and c-met has progressed nicely and downregulation of this signaling pathway showed good inhibition of tumor growth in vitro and in vivo. The knock down of SF/HGF was shown to inhibit cellular proliferation, angiogenesis, and to promote apoptosis. The data from these studies indicated that the SF/HGF c-met autocrine loop would make an excellent target for ribozyme-based therapeutics because knock down of this factor would produce pleiotropic inhibitory signals in glioma cells.

4. Hammerhead ribozyme to inhibit protein kinase Cα and DNAzyme to endostatin

Synthetic hammerhead ribozymes were used to inhibit the expression of protein kinase Cα (PKCα) in glioblastoma cells in vitro and in vivo. The ribozymes contained 2’-amino modifications and were synthesized by T7 transcription. The modified ribozymes were more stable to nuclease degradation and were transected into the glioma cells using DOTAP liposomal transfection reagent. The anti-PKCα ribozyme was able to knock down the PKCα mRNA and inhibit glioma cell growth after transfection into cell lines. The synthetic, modified PKCα ribozyme was able to inhibit tumor growth of subcutaneously-established glioma tumors in rats (Sioud and Sorensen, 1998). In vitro studies subsequently
confirmed that inhibition of PKCα caused glioma cells to enter apoptosis (Leirdal and Sioud, 1999).

To conclude this series of studies, DNAzymes were shown to have efficacy in combination with endostatin in a rat glioma model. For these experiments, the DNAzyme was designed against PKCα and shown to function by inducing apoptosis when transfected into cultured glioma cells (Sorensen et al, 2002). When combined with endostatin, the DNAzyme augmented the growth inhibition of glioma in rats bearing intracranial BT(4) C tumors compared to DNAzyme or endostatin alone. The combined treatment presented was a potentially exciting multi-modal therapeutic strategy for malignant gliomas. A multi-modal approach for treating multiple targets in the same pathway is practical as gliomas are notorious for circumventing one obstacle in a pathway (Steinbach et al, 2002; Li et al, 2003; Steinbach et al, 2004).

5. Hammerhead ribozyme to vascular endothelial growth factor

Recently a hammerhead ribozyme was expressed in a VAI pol III expression cassette. The ribozyme was targeted against vascular endothelial growth factor (VEGF) and the expression cassette was transfected into U87MG glioma cells (Ciafre et al, 2004). The VEGF ribozyme, compared to a catalytically inactive control ribozyme, effectively lowered the levels of VEGF being synthesized by the glioma cells. The ribozyme also lowered the angiogenic potential of the glioma cells as measured with an in vitro model for angiogenesis. Currently, inhibiting angiogenesis is being studied intensively as a therapeutic strategy for many types of cancers. The data from this initial study in glioma, using ribozymes to inhibit VEGF, lends further support for this gene as a target for cancer therapy.

6. Hammerhead ribozyme to target Fas ligand

Ribozymes have also been used to study the biology of T cell interactions with glioma cells. A hammerhead ribozyme, targeted against Fas ligand (Fas-L), was used to help elucidate the involvement of the Fas/Fas-L pathway in stimulating Interleukin-10 (IL-10) biosynthesis after T cells encountered glioma cells. The interesting finding was that the Fas-L expressed on the surface of the tumor induced IL-10 synthesis by the T cells but did not induce their apoptosis (Yang et al, 2003). This report provides data for a unique mechanism by which tumor cells can induce immunosuppression via the Fas pathway that does not involve apoptosis of T cells (Saito et al, 2000; Hoffmann et al, 2002).

From the studies presented here, ribozymes and DNAzymes have not only proven to be very useful for studying natural processes, but as well, for developing therapies for CNS diseases. One issue that still remains in utilizing ribozymes or DNAzymes for gene knockdown studies in vivo, in the CNS, is delivery to the tissue or cells of interest. An important distinction relative to the delivery of ribozymes versus DNAzymes is that ribozymes can be delivered to tissues or cells as genes to be expressed or as synthetic RNA molecules, whereas, DNAzymes only can be delivered as synthetic oligonucleotides.

VII. Delivery of ribozyme genes

Delivering the ribozyme as a gene and expressing it intracellularly has several advantages. First, one can obtain a sustained and stable expression of the ribozyme. Second, DNA oligonucleotides are relatively inexpensive and their use involves routine recombinant techniques. Third, their use provides some flexibility in mode of delivery, as ribozyme genes can be delivered packaged as viral vectors or simply as plasmid vector constructs. They can be administered locally or systemically to the desired cells. Local delivery has the advantage of bringing the ribozyme vectors into close proximity with the target cells, however, microcarriers or lipids likely would be needed to achieve maximal delivery of the plasmid vectors. Local delivery might be most advantageous for solid tumors, but even those tumors may have infiltrating tumor cells that may be difficult to target. Therefore, systemic delivery of ribozyme genes may be attractive, even for solid tumors, however one must factor in the dilution of the ribozyme across multiple organ systems in vivo.

For the studies mentioned in this review thus far, none involved delivery of the ribozyme genes in vivo. Cells were either transfected or transduced with the ribozyme vectors in tissue culture and then the cells were transplanted in vivo into models to study the effects of ribozyme knock down of the target genes. Therefore, when using ribozyme genes to study or treat CNS diseases in vivo, there is no clear indication of whether local or systemic delivery is more successful. The issues that would be encountered in the local or systemic delivery of either naked DNA or viral vectors carrying ribozyme genes would be similar to those encountered for delivering synthetic nucleic acid enzymes and is discussed in the next section. Besides the actual physical delivery of the ribozyme, one potential problem with delivering the ribozyme as a gene is the inability to control the level, duration and timing of the dose. Although inducible promoters are available for expressing ribozyme genes, their control is not that precise.

VIII. Delivery of synthetic nucleic acid enzymes

The delivery of synthetic nucleic acid enzymes typically requires their complex with lipids, cationic amphiphiles, or polymers to facilitate uptake by the cells of interest. Synthetic nucleic acid enzymes also usually require modifications to the phosphodiester backbone of the molecule in order to increase the resistance to nuclelease digestion. One advantage of using synthetic ribozymes or DNAzymes is to control the level and duration of the dose. Another advantage is being able to more accurately control the timing of the dose. Synthetic ribozymes and DNAzymes have been delivered to target cells by both local injection and systemic administration. While in vitro models can closely mimic what might be expected with systemic delivery, the information gained has not modeled what might be expected for delivery to the brain. This is a
result of the BBB providing an impediment for effective delivery of nucleic acid enzymes to the brain (Muldoon et al., 1999; Neuwelt et al., 1999). While methods do exist to osmotically permeabilize and transiently open the BBB, the irreproducibility of the opening and unintended damage to healthy tissue has limited the routine use of these methods clinically (Kroll and Neuwelt, 1998; Doolittle et al., 2002). Other alternative methods to traverse the BBB include transferrin and insulin receptor transport systems, which have shown some efficacy in the systemic delivery of antisense oligonucleotides to the CNS, or convection enhanced delivery (Chen et al., 1999; Demeule et al., 2002; Vinogradov et al., 2004).

For the studies described in this review, DNAzymes were delivered by systemic methods for in vitro whole tissue models and in vivo for the BT(4)C brain tumor model. The synthetic ribozymes were delivered by local injection into in vivo models for gliomas. One of the models used was a subcutaneous ectopic implantation of glioma cells in the rat and does not model nor resemble the true disease state in vivo. Significant gaps still remain in the development of efficacious delivery systems for synthetic nucleic acid enzymes to the brain.

The availability of relevant animal models is essential to not only increase our understanding of how a gene functions but to also be able to test and develop effective ribozyme based therapeutics. The 9L gliosarcoma and CNS-1 glioma, syngeneic to Fischer and Lewis rats respectively, are two models that model brain metastases or malignant gliomas (Kruse et al., 1994a; Barth, 1998). They have been extensively used by researchers for testing many types of brain tumor therapies. We have used both models to begin developing ribozyme treatments for gliomas and will present our data from preliminary studies delivering lipid complexed and naked ribozyme in vitro into glioma cell culture models as well as in vivo in the rat glioma model.

A. Preliminary data testing the cell cycle PCNA targeted ribozyme for gliomas

As mentioned earlier in the studies involving proliferative vitreoretinopathy, Immusol, Inc. (San Diego, CA) developed a chimeric ribozyme composed of RNA and DNA that specifically targeted PCNA. The ribozyme hybridizes to complementary mRNA sequences and effectively inactivates PCNA mRNA through its binding properties and by sequence specific catalytic cleavage of the strand (Figure 2). PCNA serves as an ideal target because of its crucial involvement in the elongation stage of DNA synthesis. It functions as a eukaryotic processivity factor by clamping DNA polymerase δ onto DNA. By disrupting this process with the ribozyme, the proliferation of the tumor cells may be suppressed. Indeed, we had previously demonstrated that the PCNA-targeted ribozyme, compared to scrambled or heat-inactivated ribozymes, decreased the growth of rabbit fibroblasts for the PVR model and porcine smooth muscle cells for the restenosis model (Welch et al., 1997; Mandava et al., 2002).

Since PCNA mRNA is only stable in proliferating cells/tumor cells while unstable in quiescent cells/normal brain cells, it presumably was an ideal target that would be largely unique to the glioma cells. Therefore, we hypothesized that PCNA ribozyme therapy may be able to block glioma cell proliferation without toxic effects to normal brain cells. After confirming that there was 100% homology between the targeted regions of rat and human PCNA sequences, we proceeded to effect the delivery of PCNA ribozyme to human and rat glioma cells in vitro. Lipofectin (1mg/ml) or DOTAP/Cholesterol (4mM) lipids were used to facilitate transfection of human and rat glioma cells plated at 70% confluency. The lipids were added to fluorescein-labeled PCNA-directed ribozyme (500µg/ml) for 2 hr at 37ºC in a humidified 5% CO₂ incubator. As seen by fluorescence microscopy, nearly all of the 14-07-MG glioma cells demonstrated fairly uniform cytoplasmic and nuclear dispersion of the FAM-labeled ribozyme within the cells (Figure 3).

![Figure 3](https://example.com/figure3.jpg)

**Figure 3.** Human 14-07-MG glioma cells transfected with a Lipofectin and fluoresceinated PCNA-directed ribozyme complex. Photograph was obtained by fluorescence microscopy.
To explore the potential clinical application of these treatment agents, we tested the toxicity of repeated intracranial infusions (10 µl) of the synthetic, chemically-modified ribozyme when placed into cannulated rat brain (Kruse et al, 1994b, c). In this small pilot experiment, groups of rats (n=3) were administered DOTAP:cholesterol lipid (3 mM) alone or complexed to the PCNA-directed ribozyme at low concentration (375 µg/ml). Also, the naked ribozyme was administered alone at high concentration (10 mg/ml), as was a scrambled, nonbinding ribozyme control. Animal group weights were monitored and plotted every 2-3 days before, during and after treatment (Figure 4), as we previously established that weight loss (>20%) is one indicator of treatment toxicity in non-tumor bearing rats (Kruse et al, 1993). The maintenance of weights during treatment and the increase in weights after treatment indicated there was little toxicity associated with the treatment agents administered. Animal brains were collected one week after the last of three infusions and processed for histology. Histopathological profiles of each treatment group indicated similarities between control groups and those receiving the synthetic ribozymes. From a representative ribozyme-treated brain, we conclude that minimal, focal damage occurred, as the sections 100 µm to either side of where the cannulation track appears show no evidence of damage (Figure 5A). Each successive photomicrograph shown is of the blocked inset area in the previous photograph at increasing magnification. Figure 5B shows at low power, the localized, granulomatous formation occurring at and near the instillation site of the cannula and deposition of the ribozyme. Figures 5C and 5D show necrotic debris being delimited by a fibrotic wall that is immediately adjacent to normal brain. At higher power, lymphocytes with nonactivated morphology have collected at the periphery of the granulomatous area (Figure 5E). Cells with typical mononuclear morphology appear as dense, round purple-stained cells, and some cells with a lobular nucleus indicative of polymorphonuclear morphology, are present perhaps indicating a host response to the ribozymes or to the damaged cells caused by the cannulation procedure itself. Overall, the preliminary results of the in vivo toxicity studies are consistent with only minor damage inflicted upon normal, quiescent brain cells.

In a pilot animal experiment, we tested similar animal treatment groups given the PCNA-targeted versus scrambled ribozyme, but this time in cannulated rats bearing one-week established 9L gliosarcoma (5000 cell inoculum). By digitizing stained sections directly from the...

**Figure 4.** *In vivo* toxicity assessed by mean group rat weights plotted before, during and after treatments. Groups were given intracranial administrations (days 12, 15, and 16) of DOTAP alone, or when it was complexed to the PCNA-targeted ribozyme (Rz) or scrambled ribozyme (Scr Rz) at low concentrations (375 µg/ml). The ribozymes were also administered alone at high concentration (10 mg/ml, Conc Rz and Conc Scr Rz). Intracranial infusions (10 µl) of the various treatment agents were started at one week following cannulation.
Figure 5. Hematoxylin and eosin stained tissue sections and photomicrographs taken from a cannulated rat brain receiving multiple infusions of PCNA-targeted ribozyme. Gross tissue mounts and photomicrographs of the tissue histology from a representative cannulated rat brain that had received three infusions of concentrated PCNA ribozyme. The animal brain was processed for histology at one week following the last infusion. Each successive photograph (A through E) is a higher magnification of the inset area of the previous photograph. (A) The gross mounts show minor focal tissue reaction at the site of cannula implantation but not on sections at approximately 100 µm on either side from that site. (B) The granuloma shows an area of necrosis near the bottom of the cannulation track where ribozyme was infused. (C, D) At higher power, a fibrotic wall, has formed around the necrotic tissue. (E) Lymphocytes were observed at the periphery of the wall nearby the infusion site.
slides, the tumor area as a percentage of the total brain was obtained from the section at the instillation site (Kruse et al, 2000). As well, we obtained necrosis as a percentage of the total tumor area. Representative hematoxylin and eosin-stained sections show large tumors in both the PCNA-targeted ribozyme treated brain (Figure 6A) and the scrambled ribozyme treated brain (Figure 6B). However, only necrosis/gliosis is visible in the PCNA-targeted ribozyme treated brains (pink stained area within the center of the purple tumor mass). From individual animals, we plotted the tumor area as a percentage of total brain area (Figure 7A) and tumor necrosis area as a percentage of total tumor area (Figure 7B). Measurements of areas from individual animals (●) and the treatment group medians (+) are shown. There were no significant differences in mean tumor areas between any of the PCNA-targeted ribozyme and control scrambled ribozyme treated groups. However, significant differences were detected in necrotic areas as a percentage within the tumor areas between the treatment groups as shown (p values obtained from Mann Whitney test). The PCNA-directed ribozyme induced glioma cell injury of cells it came into contact with. While these preliminary findings illustrate a localized anti-tumor effect by the experimental ribozyme, they also highlight the need to improve delivery of the synthetic nucleic acid enzymes away from the site of instillation. Convection enhanced delivery could aid in this process, as could new formulation techniques for enhancing the delivery of synthetic ribozymes to the brain (Pardridge, 1998; Chen et al, 1999; Vinogradov et al, 2004). In addition, there is a need for improvement of their delivery to the tumor cells at the invading edge of the tumor mass. Techniques for BBB modification should be explored to improve this type of delivery (Neuwelt, 1980; Black et al, 1990). Perhaps multiple administration routes should be considered, such as combining local intracranial delivery with systemic delivery. A multipronged approach to delivery might effect a better kill of the tumors centrally as well as at the leading infiltrating edge of the tumor.

B. Development of proapoptotic ribozymes for gliomas

Also in collaboration with Immusol Inc., we utilized an inverse functional genomics approach for gene discovery based on the use of a randomized hairpin ribozyme gene library. As described earlier, hairpin ribozymes cleave upstream of a GUC triplet in the target sequence. By randomizing the substrate binding sequences, we generated a library of ribozyme molecules that can potentially cleave any RNA substrate containing a GUC. The library of ribozymes was stably introduced by retroviral transduction into cancer cells that were normally resistant to apoptosis via retroviral vector transduction. Selected ribozymes that reproducibly conferred a proapoptotic phenotype were exploited to identify the gene(s) involved in this alteration. Specifically, the binding site of the hairpin ribozyme contained 16 nucleotides of unique sequence that was used to query the NCBI nucleotide sequence database by BLAST search and identify the corresponding gene. Following this protocol
Figure 7. Glioma areas and tumor necrosis areas in PCNA-targeted ribozyme treated vs. scrambled ribozyme control animals. Measurements from individual animals (black dots) and group medians (red crosses) are shown. (A) Tumor area is reported as a percentage of total brain area. There were no significant differences between ribozyme treated and control groups (Mann Whitney U). (B) Necrotic area within the tumor is reported as a percentage of tumor area. Significant differences were detected as shown (Mann Whitney U). Abbreviations: D5W, distilled H2O sham treated control; Rz, PCNA-targeted ribozyme; sRz, scrambled ribozyme; conc, concentrated.
we isolated the proapoptotic ribozymes SR6, TV2, and SR4. The corresponding genes for SR6 and TV2 were identified as a transcriptional repressor and a previously unidentified adaptor protein, respectively. The SR4 ribozyme was also derived through the phenotypic screen, however, the target for this gene is currently unknown.

We characterized the proapoptotic SR6, TV2, and SR4 chimeric ribozymes for their ability to induce programmed cell death in human glioma cell culture models. We initially tested the delivery of the SR6 chimeric ribozyme in vitro by transfection of the 13-06-MG glioma cell explant with the Gene Porter lipid (Genlantis, San Diego, CA). The cells were assayed for apoptosis by a flow cytometric procedure using 7-amino actinomycin D (7AAD) (Gomez et al, 2004; Philpott et al, 1996). 7AAD, a fluorescent dye, binds to GC regions of DNA. As cell damage progresses, the permeability of the cell membrane increases, therefore, cell uptake of 7AAD is proportional to the degree of damage (Schmid et al, 1994). We validated the method by showing that >75% of the cells within the apoptotic portion of the scattergram also express the early apoptotic marker, phosphatidylserine (Gomez et al, 2004). A scattergram profiling the injured cells, shows the combination of SR6 ribozyme and Gene Porter lipid resulted in the highest percentage of apoptotic cells compared to lipid or medium only controls (Figure 8). The percentages of cells that are live, apoptotic, or dead/necrotic as determined by this technique, show that the Gene Porter lipid treated cells showed a relatively high level of injured cells, but more were present as irreversibly-damaged necrotic/late apoptotic cells (Table in footnote to Figure 8). Since the lipid vehicle was toxic itself, we tested whether a panel of four human glioma cell explants could take up the ribozyme without it. Human glioma cells were exposed for 4 hr to the chimeric ribozymes, SR6 and TV2 in phosphate buffered saline. Then, by a quantitative in vitro morphologic assay, the adherent cells were stained with hematoxylin and eosin and scored for apoptotic cells by high power microscopy. Figure 9 shows a representative area from the control monolayer of 13-06-MG glioma cells (upper left panel) exposed to PBS (the monolayer exposed to scrambled ribozyme was similar) compared to the glioma cells exposed to the SR6 ribozyme (upper right panel). Cells with fragmented or condensed nuclei (arrows) were visible, indicating that the cells had taken up naked ribozyme, although perhaps less efficiently. Figure 9 (bottom panel) shows the percentages of apoptosis to four different glioma cell lines induced by the active SR6 and TV2 ribozymes are significantly greater compared to the scrambled ribozyme (sRz) and PBS (none) controls, all in the absence of any toxic background caused from lipid transfection agents. In order to extrapolate our in vitro findings with human glioma cells to an in vivo rat model, we wanted to demonstrate similar in vitro effects on rat 9L gliosarcoma cells with a proapoptotic ribozyme. Subsequent testing by annexin V staining for phosphatidylserine revealed that another chimeric ribozyme from the library, SR4, significantly induced cell injury of 9L cells (Figure 10). These data were confirmed with the 7AAD flow cytometric assay, where after a 3 hr exposure to SR4, 72% were apoptotic and 12% were dead/late apoptotic (data not shown). With the identification of the chimeric SR4 ribozyme as an apoptosis inducer for rat 9L cells, testing the toxicity and efficacy of SR4 administered intracranially in a rat glioma model is warranted.

**Figure 8.** Scattergrams generated from the 7-amino actinomycin D (7AAD) flow cytometric assay that provides quantitation of live (green), apoptotic (blue) and dead (necrotic/late apoptotic, red) 13-06-MG cell percentages after exposure to SR6 ribozyme (Rz) + GenePorter (left panel), GenePorter (middle panel), or medium alone (right panel). We have confirmed that a large majority of cells within the apoptotic segregated population also express the early apoptotic marker, phosphatidylserine (PS). The live, apoptotic and dead cell percentages derived from the assay for the experimental and control groups are given in the table below the scattergrams.
Figure 9. High power microscopic view of a hematoxylin and eosin stained 13-06-MG glioma cell monolayer incubated with PBS (upper left panel) versus a monolayer exposed to the SR6 ribozyme (30 µg/ml) for 4 hr (upper right panel). Apoptotic cells are readily apparent as visualized by their fragmented or condensed nuclei. From in vitro quantitative morphologic assays (300-500 total cell counts), the percentages of apoptotic cells derived from four different human glioma cell lines after 4 hr exposure to SR6, TV2, scrambled (sRz), or no ribozymes (none) are shown (bottom panel). When the treatment groups were analyzed by Mann Whitney, the experimental SR6 and TV2 ribozymes had a significantly greater apoptotic effect on the 4 human gliomas compared to the scrambled ribozyme or the PBS controls (p ≤ 0.03).

Figure 10. Apoptosis induction of rat 9L gliosarcoma cells stimulated by sham treatment, scrambled ribozyme, TV2, or SR4 ribozymes. The flow cytometric assay used fluoresceinated annexin V that targets phosphatidylserine, the early apoptotic marker, and propidium iodide. Highly injured cells with permeable membranes would be positive for propidium iodide. The percentages given for the damaged cells are based upon the cells positive for annexin V and propidium iodide.
VIII. Concluding remarks

Great progress has been made in developing nucleic acid enzymes for studying and treating diseases of the CNS. Effective targeting of ribozymes and DNAzymes has been realized. Nuclease resistant, biologically active nucleic acid enzymes are able to be routinely synthesized. However, while there are methods available for achieving adequate delivery of nucleic acid enzymes to the eye, there are still opportunities for greatly improving their delivery to the brain and target cells within it. With the availability of excellent animal models, the future is promising for developing improved methodologies for delivering efficacious nucleic acid enzyme formulations to the brain. For gliomas in particular, multiple routes of delivery, delivery of ribozymes to the brain after permeabilization of the blood-brain barrier, and pressurized local infusion intratumorally may allow better delivery of the ribozyme so that more tumor cells can be reached with these therapeutic agents.

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