Deoxyribozymes: new therapeutics to treat central nervous system disorders

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This mini-review focuses on a knockdown technology called deoxyribozymes, which has rarely been utilized in the field of neurobiology/neuroscience. Deoxyribozymes are catalytic DNA molecules, which are also entitled DNA enzyme or DNAzyme. This mini-review presents a description of their development, structure, function, and therapeutic application. In addition, information on siRNA, ribozymes, and antisense are given. Further information on two deoxyribozymes against c-Jun and xylosyltransferase (XT) mRNA are summarized of which the first is important to influence many neurological disorders and the last potentially treats spinal cord injuries (SCIs). In particular, insults to the central nervous system (CNS) such as SCI generate an inhibitory environment (lesion scar) at the injury site that prevents the endogenous and therapy-induced axonal regeneration and thereby limits repair strategies. Presently, there are no treatments available. Hence, deoxyribozymes provide an opportunity for new therapeutics that alter the inhibitory nature of the lesion scar and thus promote axonal growth in the injured spinal cord. When used cautiously and within the limits of its ability the deoxyribozyme technology holds promise to become a major contributing factor in repair strategies of the CNS.

Keywords: central nervous system trauma, brain insult, drug development, DNA enzymes, catalytic DNA, c-Jun, xylosyltransferase, proteoglycans

INTRODUCTION

Scientists have worked for over 100 years to understand the mechanisms that are involved during axonal growth and regeneration processes. In spite of considerable success no medication is available. Hence, the development of therapeutics to treat central nervous system (CNS) trauma such as spinal cord injury (SCI) is a long awaited intervention in pursuit of finding a cure for paralysis. Presently, approximately 10,000 people sustain a SCI every year in Europe; whereas approximately 40 million patients worldwide are paralyzed due to damage to their spinal cord. About 70% of all these victims sustain a contusive injury. Most of them are young men with an average age of 33.4. As a consequence about 2.5 million people must presently live wheelchair-bound or they are even worse off (Adams and Cavanagh, 2004). An early treatment to prevent long-term complications after SCI with the goal to accelerate recovery is a necessary step toward medicating CNS trauma. Hence, this review will introduce the deoxyribozyme technology as a potential therapeutic that promotes regeneration and axonal growth after insults to the brain or spinal cord. Its development, structure, function, design, and therapeutic applications will be reviewed to provide an alternative to common knockdown agents.

DEOXYRIBOZYME

Single-stranded, catalytic DNA molecules, which are also being called DNA enzymes or DNAzymes, have been reported not to exist in nature. Instead they have been developed by Santoro and Joyce (1997) who used a technique called systematic evolution of ligands by exponential enrichment (SELEX). Here, a selection process was used, in which a RNA substrate was provided to a library of approximately $10^{14}$ potential single-stranded DNA enzymes. Via enrichment of the functional catalytic DNA molecules two deoxyribozymes named 10-23 and 8-17 with a length of approximately 35 nucleotides were isolated (for review, Silverman, 2005). The catalytic loop structure of the 10-23 deoxyribozyme contains 13, the one of the 8-17 15 nucleotides, of which each is surrounded by sequence-specific binding arms (Figure 1A). To be able to digest any target mRNA and hence prevent the translation to a protein these two deoxyribozymes require divalent metal ions such as Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, or Cu$^{2+}$. These metals function as a general base or as a Lewis acid either to stabilize the transition state of the reaction and/or to assist in folding of the enzyme into an active conformation (Santoro and Joyce, 1998). An exception is the deoxyribozyme motif, Na8, which increases RNA cleavage by 108-fold in the absence of divalent metals (Geyer and Sen, 1997). Another deoxyribozyme requires
l-histidine or a closely related analog to catalyze RNA phosphoester cleavage (Roth and Breaker, 1998). Target specificity of the deoxyribozyme is controlled by the sequence of the binding arms. The binding arms of both deoxyribozymes form Watson–Crick base pair interactions of 8–12 nucleotides with their target mRNA. A phosphodiester linkage in this target mRNA is cleavage by deoxyribozymes between an unpaired purine (adenine [A] or guanine [G]) and a paired pyrimidine (cytosine [C] or uracil [U]) to an end product of 2',3'-cyclic phosphate and 5'-hydroxyl termini (Figure 1B). In case of the 10-23 deoxyribozyme the required dinucleotides in the target mRNA are G-C, G-U, A-C, or A-U whereas the 8-17 deoxyribozyme requires solely A-G as recognition sequence. The order of best to worst substrate cleavage is as follow: rAU > rGU > rGrC > rArC, whereas the relatively poor activity of a deoxyribozyme against rArC- and rGrC-containing substrate can substantially be increased by chemical modifications to the binding domain that subtly weaken the interaction with the substrate. When dG is substituted with deoxyinosine (dI) such that the three hydrogen bonds between rC–dG are converted to two hydrogen bonds rC–dl, the biological activity of that deoxyribozymes that target rRrC junctions (R = rA, rG) in the substrate is improved (Cairns et al., 2003). To prevent deoxyribozyme digestion through serum nucleases, the following modifications are the most frequently used: (1) phosphorothioated (PS) bases at the 5' and/or 3' end; (2) 3'-3'-linked inverted thymidine or any other inverted nucleotide; (3) locked nucleic acid (LNA); or (4) phosphoramidated bases (for more detail on the design of deoxyribozymes please see Achenbach et al., 2004).

Deoxyribozymes show high flexibility, specificity, and low production costs, which makes them a valuable asset for treatment of, e.g., SCI. In addition they have the advantage to be administered and internalized into cells without using transfection agents, electroporation, or infection albeit utilizing a concentration that does not induce side effects. Hence, any mRNA that participates in the inhibitory environment after insult to the CNS can serve as a substrate for digestion. The utilization of deoxyribozymes alone or in combination with other approaches has the potential to generate a growth-permissive environment at the lesion site. This holds promise to enhance axonal regeneration, and thus, increases the chances of functional recovery.

**OTHER KNOCK DOWN TECHNOLOGIES**

**SMALL INTERFERING RNA**

Presently, siRNA is the most frequently used knockdown technique in science with developments of approximately 21 potential therapeutics to a variety of diseases (Burnett et al., 2011). The mechanism of siRNA, which are double-stranded RNA molecules of usually 21 nucleotides that contain a 3' overhang of 2 nucleotide on either end, is well known and documented by a plethora of publications (see reviews: Fire, 1999; Tuschl, 2001; Mello and Conte, 2004). In addition to its role to interfere with the expression of specific genes, it also acts in antiviral mechanisms or shaping the chromatin structure in a genome. siRNA was discovered by Andrew Fire and Craig Mello, who received the Nobel Prize 2006. In light of such excitement and success it is surprising that Roche closed, at the end of last year, all R&D facilities that were working on identifying siRNAs sequences to treat various diseases and disorders. In addition Novartis terminated a contract with Alnylam, a company that provides a platform for the identification of siRNA target sequences. However, Novartis continues to work on the 31 siRNAs, which they have internally but they are not interested in indentifying new sequences. Furthermore, Pfizer and Abbott also demonstrate no further interest in this technology. Instead, Pfizer (New York) in conjunction with Santaris (Horsholm, Denmark) formed an alliance to develop an antisense strategy utilizing PS–LNAs (Pollack, 2011; Schmidt, 2011).

**RIBOZYMES**

Ribozymes (RZs), mostly used as hammerhead or hairpin RZs in therapeutic applications, are naturally occurring, single-stranded RNA molecules, which contain a catalytic loop structure and cleave their target RNA in a sequence-specific manner. They have been discovered in the 1980s for which Sidney Altman and Thomas Cech received the Nobel Prize 1989. RZs were the first demonstration that RNA is able to have catalytic/ enzymatic properties, which
lead to the belief that DNA molecules in form of deoxyribozymes are also able to digest mRNA. Based on the SciClips database 18 potential drug targets are in focus of RZ research. The recognition sequence of the hammerhead RZs contains a XUN structure, where X is any base and N is A, C, or U (Haseloff and Gerlach, 1988), whereas hairpin RZs recognize RCN/GUCB with B = C, G, or U, N = any base and R = G or A as a target sequence (Joseph et al., 1993).

ANTISENSE

The antisense technology, which was developed in the 1970s (Zamecnik and Stephenson, 1978), is the trailblazer for gene knockdown agents used in science and in particular in the nervous system. Here, a single-stranded DNA molecule, an antisense oligonucleotide (ODN), binds stoichiometrically to the target mRNA and prevents its transcription, splicing, or translation to a protein (Eckstein, 2007). If the cell contains RNase H, the RNA/DNA hybrid that forms after binding of the antisense ODN to the target pre-mRNA or mRNA is going to be digested. The first antisense drug, Vitavene, which treated cytomegalovirus-based retinitis, was approved 1998 and discontinued in 2004 as the drug’s market shrank (Jones, 2011). Approximately 10 other antisense drugs are being researched. Furthermore, the following proteins c-erbA/rev-erbA (Munroe and Lanzar, 1991), N-myc (Armstrong and Krystal, 1992), gonadotropin releasing hormone (GnRH, Wilson et al., 1995), Wilms’ tumor suppressor gene (WT1, Malik et al., 1995), insulin-like growth factor (IGF-II, Baccarini et al., 1994), hyperalgesia and allodynia (see mini-review: Gao and Ji, 2008). The transcription factor is not only essential to neuronal differentiation but is also a powerful mediator of neuronal apoptotic signal cascades involving c-Jun N-terminal kinases (JNKs, Kyriakis et al., 1994). In addition, JNK activation is strongly involved in inflammatory responses, neurodegeneration, and in the development of hyperalgesia and allodynia (see mini-review: Gao and Ji, 2008). Hence, the JNK pathway is an important therapeutic target to influence diverse neurological disorders (see review: Antoniou et al., 2011).

Table 1 | In vivo application of deoxyribozymes (modified from Isaka, 2007).

| Gene target | Disease (tissue)                          | Application              | Reference         |
|-------------|-------------------------------------------|--------------------------|-------------------|
| **GENERAL** |                                           |                          |                   |
| Erg-1       | Balloon injury (carotid artery)           | Adventitial (from outside of a vessel) | Santiago et al. (1999) |
| Ligation   | Adventitial (carotid artery)              | Adventitial              | Lowe et al. (2002) |
| Restenosis | Endoluminal (from inside a vessel)        | Endoluminal              | Lowe et al. (2001) |
| Tumor growth (breast carcinoma) | Transfection, subcutaneous, intratumoral |                          | Fahmy et al. (2003), Mitchell et al. (2004) |
| Ureteral obstruction (kidney) | Interstitial |                          | Nakamura et al. (2002) |
| Myocardial infarction | Intramyocardial |                          | Bhindi et al. (2006) |
| TGF-β       | Glomerulonephritis                        | Renal arterial           | Isaka et al. (2004) |
| c-Jun       | Neovascularization (corneal)              | Injection, intravitreal | Zhang et al. (2004), Khachigian et al. (2002) |
| Tumor growth (melanoma) | Subcutaneous |                          | Zhang et al. (2004) |
| Tumor growth (squamous cell carcinoma) | Transfection |                          | Zhang et al. (2006) |
|          | Inflammatory diseases                   | Intravitreal, intradermal, topical | Fahmy et al. (2006) |
|          | Tumor growth (breast carcinoma)           | Intratumoral             | Zhang et al. (2002) |
|          | Myocardial infarction                    | Intraperitoneal          | Iversen et al. (2001) |
|          | Myocardial ischemia                      | Intracardiac             | Xiang et al. (2005) |
|          | Myocardial infarction                    | Intracardiac             | Xiang et al. (2004) |
|          | Asthma (TH2 cells, mast cells, eosinophils, and epithelial cells) | Intranasal/Topical | Sel et al. (2008) |
|          | Sepsis (bacterial cell division)         | Systemic                 | Tan et al. (2004) |
| **NEUROSCIENCE** |                                 |                          |                   |
| Xylosyltransferase-1 (XT-1) | Spinal cord injury | Intrathecal | Grimpe and Silver (2004), Hurtado et al. (2008) |
et al., 2011). Investigations by Dass and Choong (2010) showed that the intravenous (iv) administration of a deoxyribozyme to c-Jun mRNA (Dx13) in a bolus dose or in an in utero assay has no toxic effect to blood and solid tissues in adult or fetal mice, only a slight hepatotoxicity was noted with histology.

After insult to the CNS, e.g., SCI an axonal growth inhibitory environment forms at the injury site. Proteoglycans (PgS) such as chondroitin sulfate (Cs−) PGs constitute a major group of molecules that participates in this axon growth-inhibition. They most likely exert their inhibitory effects through glycosaminoglycan (GAG)-side chains. GAG-chain synthesis is initiated through an enzyme called XT-1, which attaches a xylose to the Pg core protein. Application of a deoxyribozyme to XT-1 not only prevents glycosylation of PGs but also avoids the assembly of the Pg core protein into the extracellular matrix. As a consequence, microtransplanted and endogenous severed sensory axons are able to grow beyond the lesion site because the inhibitory substrate is reduced (Grimpe and Silver, 2004; Grimpe et al., 2005; Hurtado et al., 2008). Hence, the deoxyribozyme to XT-1 mRNA holds promise to be a contributing factor in treating CNS injuries.

Whether deoxyribozymes induce immunostimulatory activity requires elucidation. Characteristics of single-stranded DNA molecules to activate such innate immunity are the presence of a partially (7–10 nucleotides) or completely Ps backbone, a poly G tail at the 3′ end, 5′ end, or both, an internal palindrome sequence and an unmethylated CpG motif. CpG motifs induce splenic B cell proliferation, dendritic cell maturation, antibody production, activation of T lymphocytes and natural killer cells as well as cytokine production such as interleukin (IL)-6, IL-12, and interferon γ. The CpG motif is considered a pathogen-associated molecular pattern, which is recognized by Toll-Like Receptor 9 (TLR9). TLR9 is constitutively expressed only in B cells and plasmacytoid dendritic cells in humans and other higher primates (Krug et al., 2001). Such CpG motifs are likely to occur in deoxyribozymes.

However, no information is available on the immunological status of deoxyribozymes. Explicit information on the half-life of deoxyribozymes in the cell or after systemic administration is also not available. However, extensive investigations on antisense PTOs revealed that their plasma half-life after iv administration lies between 30–85 min (Stevenson et al., 1999, see review: Wang et al., 2001) whereas within the cell contains their lifespan approximately 2–3 days.

The steady success of the deoxyribozyme technology to generate potential therapeutics funneled into the foundation of two start up companies, the US-based CytoGenix Inc. (Tian et al., 2004) and the Germany-based Sterna Biologicals (Sel et al., 2008), which tests DNA enzymes in preclinical trials of cancer, sepsis, and asthma research.

CONCLUSION

All above mentioned knockdown technologies are incapable to pass the blood–brain or spinal cord–barrier based on their charged DNA or RNA backbone. Modifications such as PTOs, PNA, and LNA are utilized to make up for this deficit. In addition, they prevent exonuclease degradation and increase stability. To direct any of the molecules to specific regions of the body, e.g., a tumor, is an equal challenge to all aforementioned knockdown agents, whereas uptake into cells without any aid is in favor of single-stranded molecules. All together one can say each of the knockdown techniques has its pros and cons depending on scientific demands or personal preference. Hence, each agent needs to be evaluated from case to case. There might not even be one technology to favor but at the same time, none of these techniques should be overlooked either.

ACKNOWLEDGMENTS

Research on deoxyribozymes in the Grimpe laboratory has been supported by the German Research Foundation (DFG), Forschungskommission der HHU, Wilson Medical Research Foundation, and Glaucoma Foundation.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 20 June 2011; accepted: 02 September 2011; published online: 23 September 2011.