REVIEW

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C-jun: pharmaceutical target for DNAzyme therapy of multiple pathologies

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Recent studies have demonstrated the potential of DNAzymes for therapy of various diseases via mRNA target-specific cleavage. One such target, the basic region-leucine zipper protein c-Jun, has been targeted and efficacy seen in such pathologies as cancer, ocular neovascularisation, arterial thickening, acute inflammation, and rheumatoid arthritis. This review discusses these cases in turn, and presents some new data on the applicability of a c-jun DNAzyme against a panel of cancer cells. Importantly, downregulation of c-jun is noted to cause apoptotic death of cancer cells. These studies collectively demonstrate the potential of this DNAzyme as a lead candidate for DNAzyme therapeutics.

1. Introduction

Over the past decade, the human genome sequencing project, and more accessibility of gene microarray tools have dramatically altered the pace with which pathological specimens are analysed for alterations in the expression of genes on a global scale. Hand-in-hand with these findings, we now possess the capacity to selectively attenuate the expression of specifically targeted genes both as a means of dissecting molecular function as well as switching genes down or off in attempting therapy.

Strategies to specifically knockdown gene expression have recently received considerable attention, and these include antisense, oligodeoxynucleotide decoys, siRNA, aptamers, and the catalysts – ribozymes and DNAzymes. Technologies for gene modulation, once items of limited potential, have now given way to an oligonucleotide-based therapeutic. Approved by the FDA in 1998 was Isis Pharmaceuticals' Vitravene (fomivirsen), a treatment for cytomegalovirus retinitis (CMV) in immunocompromised patients, such as those with AIDS (Marwick 1998).

On the downside, it has been nearly a decade since the approval of Vitravene, and there has been a relative paucity of such drugs emerging through the drug discovery and development pipelines of pharmaceutical companies. However, the ability to specifically target gene expression in such a novel way means that if a potent molecule is found, then it may well be useful against various pathologies as is the case with a c-jun DNAzyme discussed below.

2. DNAzyme overview

Through Watson-Crick-based interactions with complementary sequences mediated by such attributes as flexible binding and discrimination of nucleic acid substrates, DNAzymes perform catalytic reactions with great precision. These molecules, discovered in 1994 (Breaker and Joyce 1994), combine the benefits of highly sequence-specific ribonuclease-independent RNA destruction, with the relatively stable chemistries employed in oligodeoxynucleotide-based antisense reagents.

Essential criteria dictating the usefulness of a DNAzyme are listed as a summary in Table 1. The most well characterised DNAzyme is the "-23" subtype comprising a cation-dependent catalytic core of 15 deoxyribonucleotides

Table 1: Requirements for a successful DNAzyme

DNAzyme should specifically cleave its target mRNA
The target gene should play a key role in the disease process
If knocked down, the target gene's role should not be compensatable by other genes
Target gene inhibition should not adversely influence normal physiological processes
Capable of being carried and delivered by a variety of agents, if free delivery is suboptimal
Is amenable to chemical modification to avoid degradation
Should not in any form cause non-specific (off target) effects

which binds to and cleaves its target RNA between an unpaired purine and paired pyrimidine through a de-esterification reaction. The catalytic core is flanked by complementary binding arms of 6–12 nucleotides in length which specifically bind to target mRNA. Once the mRNA target is bound, it gets cleaved.

In vitro cleavage experiments have shown that the 10–23 DNAzyme is highly specific and in fact quite sensitive to small changes in target sequence (Cairns et al. 1999). Imperatively, DNAzyme activity is dependent on the prevailing secondary structure of long target RNA at the cleavage site. For this reason it is important to test a range of synthesised molecules to identify those that display a high level of activity against biologically relevant target molecules. From experience (Fahmy et al. 2003; Dass 2004), the transition from in vitro cleavage kinetics to cell culture assessment (cellular and nuclear uptake, downregulation of the target gene, plus phenotypical changes) to evaluation in vivo (in clinically-relevant models demonstrating pharmacokinetics and pharmacodynamics) dramatically culls the number of DNAzymes down to a few positive ones.

While no studies to date have been performed in humans, several investigations performed in animal disease models have proven the potential applicability of DNAzymes in general (reviewed by Bhindi et al. 2007). Most of the studies have been performed to prove whether these catalytic molecules can perform their function in vivo, and thereby extend their usefulness beyond the cell culture stage. Although *in vitro* assessment establishes uptake efficiency and sequence-specificity (Sun et al. 1999; Dass et al. 2002), and is an expedited form of screening, important issues such as biodistribution, metabolism, toxicity and elimination can only be evaluated in vivo. In the past five years, considerable progress has been made with these entities. One such commonly tested entity is Dz13, the DNAzyme specifically targeting c-jun mRNA.

3. The discovery and activity of the c-jun DNAzyme – Dz13

C-jun expression has been implicated in pathological angiogenesis in cancer (Folkman 2004) and possible other diseases involving endothelial cells and vascular dysfunction. Recently, it has been shown to be directly involved in cancer initiation and progression (Franchi et al. 1998; Papachristou et al. 2003; Aoyagi et al. 1998; Tiniakos et al. 2006; Tiniakos et al., 1994; Gee et al. 2000). Thus, c-jun silencing or knockdown may prove to be beneficial against a variety of disorders.

The sequence for Dz13 was first derived at the University of New South Wales, Sydney (Khachigian et al. 2002). This was one in a series of DNAzymes designed against sites along the mRNA of c-jun, at regions of low free energy. The panel of synthesised DNAzymes were evaluated for their capacity to cleave ^{32}P-labeled *in vitro* transcribed c-jun RNA. One of the active DNAzymes, Dz13, targeting the G^{1311}U junction (where the translational start site in human c-jun mRNA is located at A^{1261}UG), cleaved the transcript within 15 min in both a time- and dose-dependent fashion, generating 474- and 194-mer degradation products. DNAzyme Dz13scr, in which the hybridizing arms of Dz13 were scrambled without altering the catalytic core, failed to cleave the substrate.

Serum-inducible c-Jun immunoreactivity as determined by Western blotting was strongly inhibited by Dz13, whereas its scrambled counterpart failed to demonstrate a pertubation in protein signal. A summary of cell culture results to date with Dz13 is provided in Table 2. Noteworthy is the finding that even at concentrations as low as 50nM, Dz13 is capable of downregulation and consequent cellular changes. Such low concentrations highlight the latent potency of this construct. In addition, we now provide new data with various sarcoma cells lines that Dz13 downregulation of c-jun in these cells can cause apoptosis (Fig. 1).

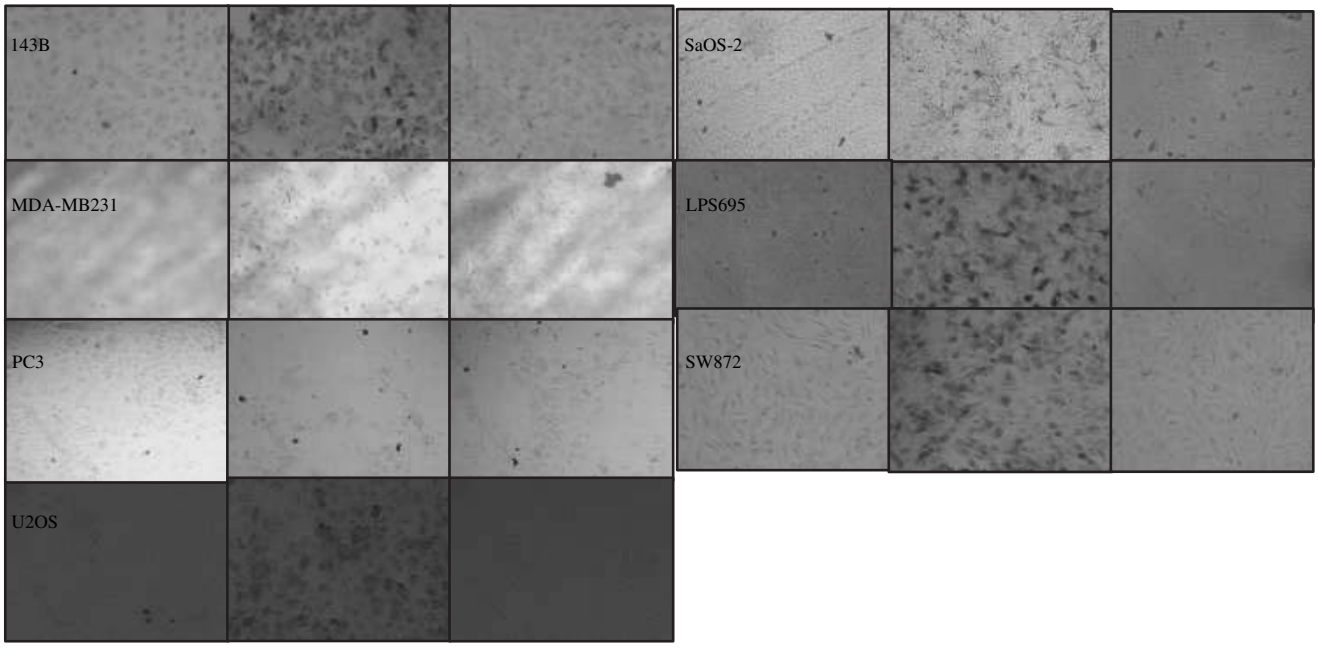

Fig. 1: Apoptosis caused by c-jun DNAzyme in human cancer cells. PC3, prostate cancer, MDA-MB231, breast cancer, 143B, U2OS and SaOS-2, osteosarcoma, SW872 and LPS695, liposarcoma. For the image panel of each cell line, the left image depicts untransfected cells, middle depicts Fugene-6-transfected Dz13 at 0.4 μM, and right panel depicts Fugene-6-transfected Dz13Scr at 0.4μM. Cells were analysed 24h post-transfection. Images were acquired under high power field, 100x magnification

Table 2: Activity of Dz13 against cultured cells

Cell line	Transfectant	Dose (μM)	Molecular and cellular changes	References
HASMC	Fugene-6	0.5	60% decrease in c-jun protein. 60% decrease in proliferation Inhibition of regrowth in denuded zone in scraping assay by 90%	Khachigian et al. 2002
HMEC-1	Fugene-6	0.2	60% decrease in proliferation	Rivory et al. 2006
RSMC	Fugene-6	0.2	85% decrease in proliferation	Rivory et al. 2006
HMEC-1	Fugene-6	0.1	75% decrease in proliferation	Goodchild et al. 2007
ARPE-19	Fugene-6	0.1	75% decrease in proliferation	Goodchild et al. 2007
HMEC-1	Fugene-6	0.05	75% decrease in adhesion of cells to monocytes	Fahmy et al. 2006
LK2 (SCC)	Fugene-6	0.2	70% decrease in c-jun protein. 60% decrease in proliferation	Zhang et al. 2006
T79 (SCC)	Fugene-6	0.2	40% decrease in c-jun protein. 60% decrease in proliferation	Zhang et al. 2006
HMEC-1	Fugene-6	0.4	50% decrease in c-jun protein. 75% decrease in proliferation Inhibition of regrowth in denuded zone in scraping assay by 50% 60% inhibition of tube formation. Reduces MMP-2 activity by 45%	Zhang et al. 2004
bEND-3	Fugene-6	0.4	60% decrease in c-jun protein. 70% decrease in proliferation	Zhang et al. 2004

Abbreviations:
ARPE, human retinal pigmented epithelial cell, bEND, brain microvascular endothelial cell, HASMC, human airway smooth muscle cell, HMEC, human dermal microvascular endothelial cell, RSMC, SV40-transformed rat smooth muscle cell, SCC, squamous cell carcinoma

4. *In vivo* proof of Dz13 activity – overview of studies

The seminal proof of *in vivo* activity of Dz13 was a demonstration that it was capable of reducing intimal thickening in injured rat carotid arteries in vivo (Khachigian et al. 2002). A dose of 0.75 mg Dz13 was complexed with Fugene-6, and mixed together with $MgCl_2$ and Pluronic gel around the injured vessel. The solution gelified and the vessels were analysed 21 days post-treatment. Interestingly, this study highlighted the usefulness of such a hydrogel system for local administration of DNAzyme therapy. In a more recent study (Murrell et al. 2007), local delivery of 0.5 mg Dz13 via balloon catheter to injured arteries in rabbits reduced intimal hyperplasia, increased vessel lumen size, and abrogated the effect of low flow on restenosis after angioplasty.

DNAzyme activity is however not limited to regulation of the vasculature. Zhang et al. (2004), demonstrated that

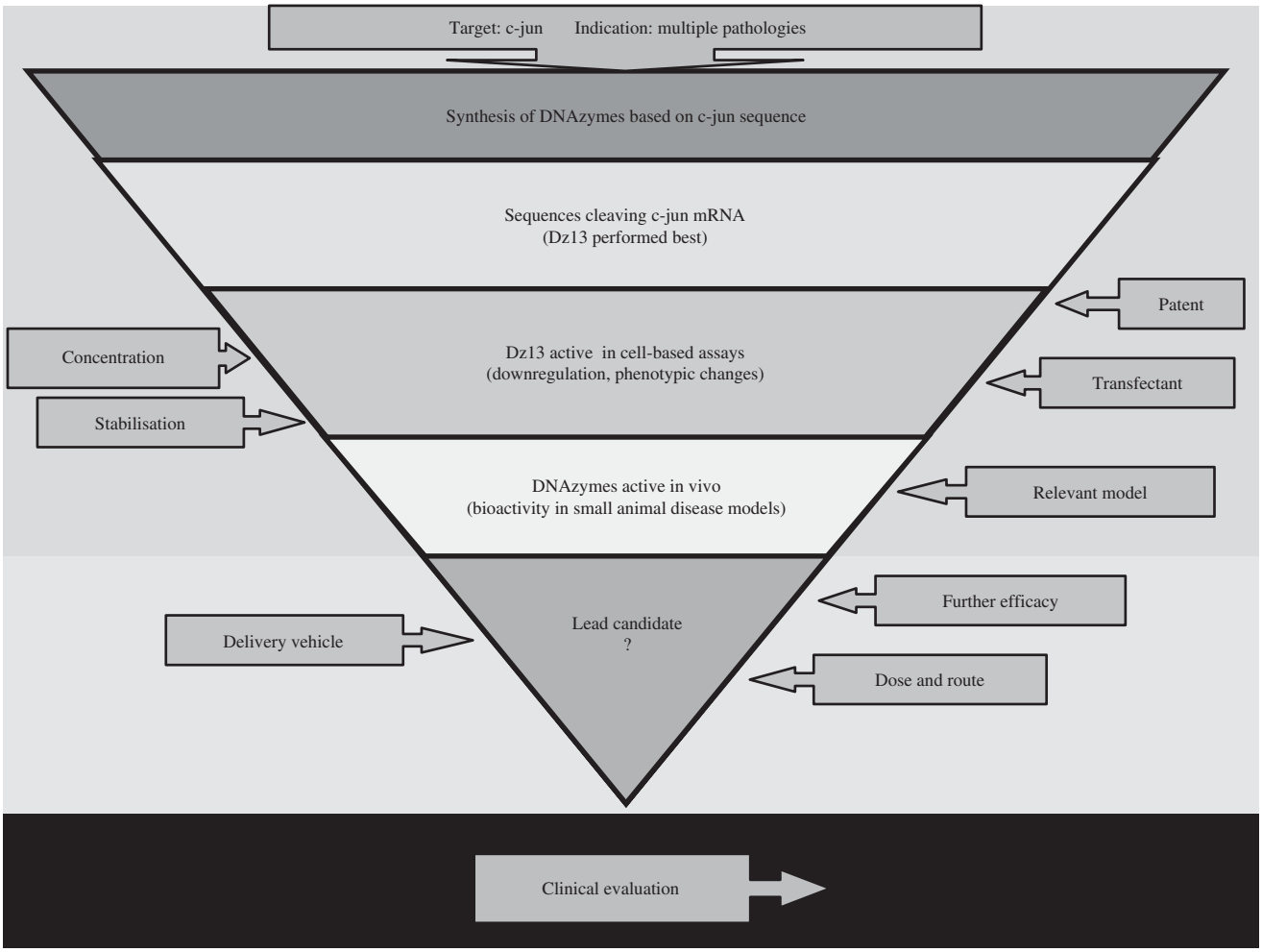

Fig. 2: Historical development and status quo for Dz13

Dz13, when complexed with Fugene-6 and coadministered with tumour cells inhibited VEGF-induced neovascularization in the rat cornea and B16 melanoma growth in mice. There was a 2.5-fold decrease in tumour volume by Dz13 and a 60% decrease in the number of tumour-associated blood vessels in these mice. The decrease in corneal vascularisation due to VEGF stimulus was 75% in the Dz13 group of rats.

Dz13 is capable of reducing not only solid melanoma growth, but squamous cell carcinoma (SCC) growth in mice as well (Zhang et al. 2006). Dz13 downregulated c-Jun expression in T79 cells and also inhibited their growth in a dose-dependent manner. In nude mice, when Dz13 was co-injected with T79 cells subcutaneously into the midback, tumour volumes in the Dz13-treated group decreased by 80%, and the blood vessel number in examined tumour sections were reduced by 40%. Importantly, the levels of c-Jun were significantly reduced by Dz13.

The function of this DNAzyme also extends to control of inflammation at various levels. Retinal neovascularisation was inhibited in mice with a single intravitreal administration of 20 μg of Dz13 (Fahmy et al. 2006). Delivery of DNAzyme to the vascular endothelial lining was confirmed. Furthermore, in a model of passive cutaneous anaphylaxis, a single local dose of 100 μg of Dz13 inhibited the vascular response in mouse ears by 70%. Delivery of DNAzyme was confirmed to the endothelium. When Dz13 was tested in a a vascular permeability (Miles) assay, 100 μg of oligonucleotide delivered locally was capable of reducing leakage by 80%.

In a model of inflammation in the rat mesenteric microcirculation, a topical delivery of 35 μg of Dz13 reduced IL-1β-induced leukocyte flux, adhesion and extravasation in mesenteric venules (Fahmy et al. 2006). Importantly, Dz13 was capable of reducing expression of a variety of molecules involved in all stages of the inflammatory process such as vascular cell adhesion molecule (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). Dz13 also suppresses inflammation and bone erosion in a murine model of rheumatoid arthritis. Both joint thickness and synovial inflammatory cell infiltration were reduced with a 50 μg dose of Dz13 administered intraarticularly. Dz13 also showed efficacy in a lung sepsis murine model. Doses of 100–200 μg Dz13 were administered into the lung via the nares of mice.

Thus, the above studies, using relevant models for human disease in animals, demonstrate the ability of Dz13 to attenuate disease progression across various pathologies. Some rather smart delivery vehicles as hydrogels and devices such as balloon catheters have been employed for local, regional administration. One true test is whether a more systemic delivery approach, such as that administered intravenously or intranasally leads to long-lasting benefits. Such studies will pave the way for future progress of this molecule towards clinical evaluation (Fig. 2).

5. Summary

Albeit DNAzyme technology is in its relative infancy, progress thus far suggests the emergence of an exciting era of gene modulation, one that may be able to dramatically change bioactivity *in vivo*, and even be used to control molecular and cellular interactions relevant to disease and fill an unmet need where conventional therapies are either ineffective, toxic, or both. One such strong candidate for this class of potential therapeutics is Dz13, the DNAzyme that downregulates c-jun. The studies discussed above attest to the latent potential of this DNAzyme against a variety of pathologies where c-jun knockdown is required.

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