DNAzyme Delivery Systems: Getting Past First Base

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DNAzyme technology has evolved into a discipline with the potential for presenting drug agents against cancer and atherosclerosis. However, current approaches still rely on sub-optimal drug delivery systems (DDSs) for DNAzymes. Certain DDSs have shown potential, such as chitosan and polyethylenimine (PEI), although more emphasis needs to be placed on actual efficacy and safety, in addition to establishing the pharmacokinetics of the molecule being tested. Unfortunately, the plethora of DDSs reported for antisense delivery – the trailblazer for target gene knockdown agents – have yet to yield even one entity capable of being used clinically, and clinicians have resorted to administering continuous systemic free oligonucleotides with promising, albeit lukewarm results. The challenge ahead for DNAzymes to be considered genuine drug candidates alongside siRNA and antisense simply lies in the better implementation of DDSs.

Keywords: chitosan, DNAzyme, drug delivery system, gene therapy, liposome, oligonucleotide

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1. The characteristics and development of DNAzymes

Deoxyribozymes (DNAzymes), first discovered in 1994 by Ronald Breaker and Gerald Joyce [1], are single-stranded, synthetic DNA catalyst engineered to comprise a cation-dependent catalytic core of about 15 deoxyribonucleotides (**Figure 1**). The DNAzyme construct binds to complementary sequences through Watson–Crick base pairing and cleaves target messenger RNA (mRNA) in an RNase-independent manner [2]. mRNA cleavage occurs through de-esterification between an unpaired purine and paired pyrimidine and is supported by the large availability of such sites in the secondary structure of the target mRNA's own intramolecular base pairing [3]. The most well-characterised subtype is the '10 – 23 subtype', which has emerged as a potential new class of nucleic acid-based drugs because of its relative ease of use, the low cost of synthesis, its stability and flexible yet rational design features [4]. Sequence specificity of DNAzymes is determined by the order of deoxyribonucleotides in the hybridising arm of the DNAzyme, while its activity is dependent on the prevailing secondary structure of long target RNA at the cleavage site [5]. *In vitro* selection assays also help to select for DNAzymes which have been designed to maximise the cleavage efficiency of RNA substrates, producing DNAzymes with high turnover rates and substrate affinities [5].

DNAzymes were first tested against cancer cells by Wu *et al.* about a decade ago [6]. Three DNAzymes were designed against two variants of the proto-oncogene p210 *bcr-abl* gene and the p190 variant. DNAzymes specifically cleaved and inhibited p210 *bcr-abl* protein expression in chronic myeloid leukaemia K562 cells by almost 40% and inhibited cell growth by more than 50% over a five-day period, suggesting the efficiency of specific DNAzymes towards the downregulation of appropriate genes and poising DNAzymes as potential genotherapeutic molecules. Another DNAzyme, designed against the urokinase-type plasminogen activator

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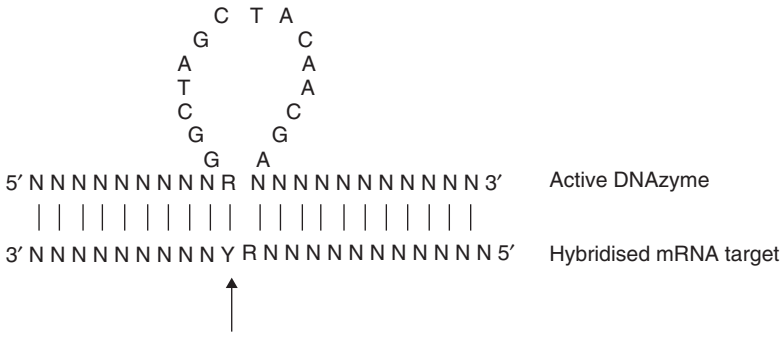

Figure 1. Schematic of a 10-23 catalytic motif DNAzyme. DNAzyme (top strand) cleaves target mRNA at the position indicated by the arrow. R=A or G, Y= U or C.

(uPA) receptor (uPAR, implicated in signal transduction and biological processes leading towards cancer metastasis and angiogenesis), was similarly able to decrease uPAR mRNA and protein concentrations, resulting in suppressed osteosarcoma cell invasion [7]. In addition, DNAzymes designed against different cancer target genes such as *c-Jun*, *Egr-1* and vascular endothelial growth factor (VEGF) have been designed. DNAzymes designed against the early growth response (*Egr-1*) mRNA were able to block angiogenesis in mice and also inhibit MCF-7 human breast carcinoma growth in nude mice. In addition, *Egr-1* DNAzymes inhibited the endothelial expression of downstream pro-angiogenic fibroblast growth factor-2 (FGF-2), but not that of anti-angiogenic VEGF, allowing for suppressed tumour growth without influencing other factors like body weight, wound healing, haemostasis or reproduction [8].

Egr-1 DNAzymes also displayed efficiency in breast carcinoma cells through the sequence-specific inhibition of *Egr-1* protein expression and its resultant anti-tumourigenic processes [9]. The *Egr-1* DNAzymes inhibited breast carcinoma cell migration and chemoinvasion *in vitro* and were also efficacious against solid breast carcinoma growth in athymic nude mice. DNAzymes have also been applied in other disease indications such as atherosclerosis [10]. One DNAzyme was designed against the basic region–leucine zipper protein family (bZIP) transcription factor *c-Jun*, which was found to be transiently induced subsequent to arterial injury in animal models and shown to be expressed in human atherosclerotic lesions. The *c-Jun* DNAzyme efficiently inhibited *c-Jun* protein expression in vascular smooth muscle cells at a potency exceeding that of an exact non-catalytic antisense oligodeoxynucleotide equivalent [10].

2. Problems and drawbacks

In keeping with the pursuit of more effective genotherapeutic molecules, an earlier study by Cieslak in 2002 looked at modifications to the conventional '10 – 23' deoxyribozymes for differences in kinetic parameters [11]. The authors subsequently found that DNAzymes modified through the introduction of two phosphorothioate linkages or two 2′-O-methyl-substituted residues at both the 5′ and 3′ ends significantly enhanced DNAzyme resistance to nucleases present in the blood while still retaining its potency against target mRNA. Such phosphorothioate modifications, although attempted for DNAzyme stabilisation, are more frequently applied in antisense oligonucleotides. However, they are not without toxicity [12], immunological side effects [13] and non-specific interactions with cellular membrane proteins [14]. In addition, phosphorothioate modifications add more negative charge to DNAzymes and decrease the binding affinity for target mRNA, thereby decreasing its catalytic potency [4,5]. Thus, phosphorothioate modifications are now less favoured over 3′-3′ inverted thymidine modifications (**Figure 2**). The 3′-3′ inversion modification increased DNAzyme stability and catalytic activity by up to 20-fold compared to the unmodified form [4].

Another development in DNAzyme design is that of the locked nucleic acid (LNA). LNAs have been attractive monomers for modifying DNAzymes in efforts to increase binding affinity [15,16]. Locked nucleic acid bases comprise of a 2O-4C methylene bridge that locks in a C3′-endo conformation, effectively increasing affinity for complementary sequences [17,18]. The advantages of LNA include increased thermal stability of duplexes towards complementary DNA or RNA, resistance against 3′-exonucleolytic degradation and greater solubility due to structural similarities to nucleic acids. LNA incorporation into DNAzymes, however, has influenced both the catalytic activity and biological potency of DNAzymes [15]. A more recent attempt to improve DNAzyme stability is the introduction of hairpin DNAzymes, where stem-loop hairpins are added to the end of the substrate-bindings arms. Hairpin DNAzymes displayed resistance to nucleolytic degradation for up to three days after transfection and produce better gene knockdown than non-hairpin DNAzymes with the same catalytic domain [19]. No non-specific effects or cytotoxicity have been observed to date with these agents, although more rigorous testing is required.

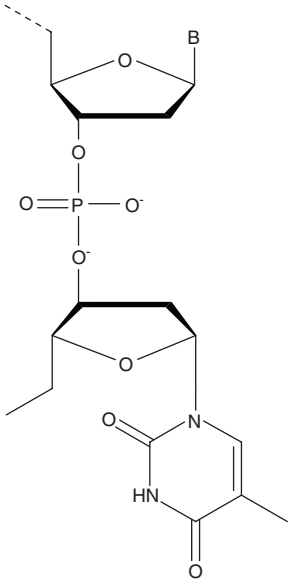

Figure 2. 3′-3′ inverted thymidine. The phosphodiester linkage is connected between the 3′-positions of each nucleotide. B = base

Having shown to be efficient both *in vitro* (**Table 1**) and *in vivo* (**Table 2**), what now remains is for DNAzymes to be delivered selectively and maybe even specifically to target tissues in the body. It is worthwhile noting that most DNAzyme studies have been attempted on cancer and cardiovascular disease models, crippling diseases where better treatment strategies are sought. As the main concern regarding the therapeutic use of DNAzymes and most other genotherapeutic molecules revolves around its distribution after administration, delivery systems were developed to assist in the delivery of DNAzymes. Ideally, these delivery systems would function to carry DNAzymes from the site of administration, either through the bloodstream or tumour stroma, to the organ or tumour and act directly on the diseased cells (for example cancer cells). The DNAzymes must finally enter the nucleus, locate the target mRNA and finally annul its message. In a study conducted by Khachigian *et al.*, Fugene-6 transfection agent (Roche Applied Science, Australia) was used as the delivery system of *c-Jun* DNAzymes *in vitro*, although the report did not comment on any side-effects from the chosen delivery system. Fugene-6 is meant for *in vitro* usage and needs to be replaced as a vehicle for future *in vivo* studies [10].

Thus, central to the application of DNAzymes as potential therapeutic agents, it is highly desirable that DNAzymes possess certain criteria such as: i) specifically cleaving target mRNA, which should play a non-compensatable role in the disease process; ii) being amenable to chemical modification to avoid degradation; iii) should not cause non-specific effects which might also adversely influence normal physiologic processes; and iv) should be capable of being carried and delivered by a variety of suitable vehicles in situations where free delivery of DNAzymes is sub-optimal. This review will focus extensively on DNAzyme delivery strategies.

3. DNAzyme delivery systems – past to present

As the DNAzyme class of molecules are a new genotherapeutic entity that has only been explored more recently, there is a lack of DNAzyme delivery systems for these entities. In fact, the development of DNAzyme delivery systems only started six years ago.

Novel attempts at DNAzyme delivery systems first involved the encapsulation of DNAzymes within co-polymers of poly(lactic acid) and poly(glycolic acid) (PLGA) microspheres using a double emulsion–deposition method [34]. The DNAzymes, bearing phosphorothioate modifications, were designed against human epidermal growth factor (EGFR) mRNA and investigated for its *in vitro* drug release profile after encapsulation into the PLGA microspheres. The release profiles of these DNAzymes were described as biphasic, characterised by an initial 'burst effect' release over five days, followed by a second phase of sustained release over the next 40 days [34]. This two-step release system was attributed to the genotherapeutic construct being present at or near the surface of the microspheres during the initial phase, while the delayed second phase was thought to be the result of drug movement from within the deeper matrices of the polymer through pores or networks [34]. This preliminary attempt at DNAzyme encapsulation showed potential for the design and usage of delivery systems in order to achieve sustained DNAzyme release and accumulation. However, this study failed to take into account other factors such as the amount of DNAzyme initially encapsulated into the microspheres and the activity of the DNAzymes following release.

A different attempt at DNAzyme delivery systems involved the complexation of cyclodextrin-containing polycation (CDP) with DNAzymes, designed against the c-*myc* proto-oncogene, via electrostatic interactions to form uniformly sized 100 nm particles termed polyplexes [35]. Polyplexes were formulated through the end-modification of cyclodextrin-based polycation with imidazole (CDP-Imid), used as a nucleic acid condensing agent, adamantane-poly(ethylene glycol) (AD-PEG) as a particle stabiliser, adamantane-diglutamate-poly(ethylene glycol)-galactose (AD-gluglu-PEF-gal) to neutralise excess cationic charges on the polyplex surface and adamantane-poly(ethylene glycol)-transferrin (AD-PEG-Tf), which imparts tumour-targeting specificity to the polyplexes via the transferring ligand. DNAzymes were completely loaded into the CDP-Imid polyplexes at different molar charge ratios (polymer : nucleic acid) and the polyplexes were subsequently internalised efficiently and rapidly into cells; DNAzymes were also visualised within the cells after 15 min of exposure. In contrast to free DNAzymes which could not enter cells, DNAzymes were packaged and protected in

Table 1. DNAzymes – Short overview of *in vitro* usage.

mRNA target	Pathogenesis	DNAzyme usage	Results/Activity	Ref.
Bcr-abl	Chronic myeloid leukaemia (CML), acute lymphoblastic leukaemia (ALL).	Three DNAzymes were designed against two variants of the p210 *bcr-abl* gene and p190 variant. DNAzymes were transfected into CML K562 cells using GS2888 cationic liposomal reagent.	Inhibited p210 *bcr-abl* protein expression by K562 cells by ~ 40% and inhibited cell growth by > 50% over six days. In a test on freshly isolated $CD34^+$ bone marrow cells from CML patients, transfected DNAzymes specifically inhibited the growth of *bcr-abl* –positive colonies by 50 – 80%.	[7]
β_1 and β_3 integrin	Important in cell–cell and cell–matrix interactions in the pathogenesis of various tumours.	Two DNAzymes to β_1 and β_3 mRNA were designed to contain a 15-deoxynucleotide catalytic domain flanked by two substrate recognition segments of 8 and 10 deoxynucleotides for β_1 and β_3 DNAzymes, respectively. DNAzymes were partially modified with phosphorothioate and with 2′-O-methyl groups at both the 5′ and 3′ ends.	DNAzymes in the presence of Mg^{2+} specifically cleaved their ubstrates – β_1 and β_3 synthetic mRNA fragments. DNAzymes abolished microvascular endothelial cell capillary tube formation in fibrin and Matrigel, signalling usefulness against tumour-induced angiogenesis. Modified DNAzymes were significantly more potent than the unmodified DNAzymes due to higher resistance to nuclease degradation Phosphorothioate analogues of the DNAzymes significantly inhibited expression of β_1 and β_3 integrin subunits in endothelial cells and K562 cells at the level of mRNA and protein synthesis.	[11]
Epidermal growth factor receptor (EGFR)	Receptor tyrosine kinase of the Erb B receptor family that is abnormally activated in epithelial tumours.	DNAzymes targeting the EGFR mRNA.	Inhibited the growth of EGFR over-expressing A431 cancer cells in a dose-dependent manner when delivered by cationic lipids. Effects of cell growth were correlated with concomitant dose-dependent reduction in EGFR protein expression.	[20]
PML/RARα fusion gene	Acute promyelocytic leukaemia (APL).	DNAzymes (Dz1 and Dz3) were designed to cleave the PML/RARα transcript at the GC nucleotides at the fusion point and three nucleotides upstream of that respectively. Unique quadruple cleavage activity of designed DNAzymes.	DNAzymes cleaved PML/RARα mRNA efficiently and specifically, resulting in ~ 80% downregulation in protein levels. Cell-based assays using APL cells showed DNAzymes inhibited proliferation and induced apoptosis in these cells.	[21]
Latent membrane protein (LMP1)	Encoded by Epstein–Barr Virus (EBV) implicated as a major oncogenic factor in EBV-mediated carcinogenesis.	DNAzymes targeting LMP1 were transfected into B95-8 cells (established from peripheral blood lymphocytes of a Marmoset monkey and releasing high titres of EBV), which constitutively express LMP1. Tetramethylpyridyl porphyrin was used as a transfection agent to attain reasonable transfection efficiency.	Significant downregulation of LMP1. Consequent inhibition of B95-8 cell growth at the $G_0 - G_1$ checkpoint. Concomitant downregulation of *bcl-2* gene expression, suggesting an association between LMP1 and apoptosis. Induction of cytochrome *c* release from mitochondria further confirmed apoptosis.	[3]

Table 1. DNAzymes – Short overview of *in vitro* usage (continued).

mRNA target	Pathogenesis	DNAzyme usage	Results/Activity	Ref.
Human survivin in PANC-1 cells	Human pancreatic carcinoma – regulates cell division and inhibits apoptosis.	Anti-survivin mRNA DNAzyme designed. Transfected into PANC-1 cells through liposomes.	DNAzyme cleaved surviving mRNA efficiently in a dose- and time- dependent manner. Achieved ~ 80% decrease in target protein levels 72 h after a single transfection. Pushed the cells into apoptosis and inhibited the growth of PANC-1 cells.	[22]
Urokinase-type plasminogen activator receptor (uPAR)	Implicated in signal transduction and biological processes including cancer metastasis, angiogenesis, cell migration and wound healing.	Three different DNAzymes (Dz372, Dz483, Dz720) targeted against three separate purine and pyrimidine junction in uPAR mRNA.	Decrease target uPAR mRNA transcript *in vitro* by ~ 80%. Downregulate uPAR mRNA in human osteosarcoma SaOS-2 cells after transfection. One DNAzyme (Dz720) decreased uPAR mRNA within four hours of transfection and inhibited uPAR protein concentrations by 55% in SaOS-2 cells, resulting in reduced SaOS-2 cell invasion in Matrigel.	[7]

polyplexes which allowed intracellular uptake into three different cells lines: cervical carcinoma HeLa cells, ovarian carcinoma A2780 cells and colorectal carcinoma HT29 cells.

For specific delivery and retention of DNAzymes within the tumour, the authors incorporated transferrin as a targeting ligand and a fluorescent tag for visualisation in mice. Three types of polyplexes were prepared and imaged using transmission electron microscopy: unmodified polyplexes, PEGylated polyplexes (PEG-polyplex) and transferrin-modified PEGylated polyplexes (Tf-PEG-polyplex). The polyplexes appeared as condensed, uniformly sized spherical particles with diameters ranging from 30 to 50 nm. Tf-PEG-polyplexes were then injected intraperitoneally (i.p.) with free DNAzymes as a control into different mice groups. Whole body imaging (WBI) results revealed a high fluorescence and large concentration of DNAzymes in the tumour, liver and peritoneal cavity eight hours post-injection of Tf-PEG-polyplexes treated mice. Significant levels of polyplexed-DNAzymes were still observed in the tumours of mice 24 h post-injection, while no fluorescence was detected in mice injected with free DNAzymes. Therefore, this shows that free DNAzymes diffuses rapidly throughout the body, while polyplexed-DNAzymes concentrates and is retained in several target organs (tumour, liver and kidney). Intravenous administration of the Tf-PEG-polyplexes also demonstrated highly efficient delivery of therapeutic constructs throughout the animal, as very little fluorescence was retained at the injection site. Similar to results obtained following i.p. administration, intravascular injection of both DNAzyme types after 24 h revealed the distribution and retention of Tf-PEG-polyplexes in the target organs, while no free DNAzymes were detected. In addition, intravascular injections presented as a better administration route, as DNAzymes delivered in Tf-PEG-polyplexes were internalised by tumour cells, while those delivered via i.p. injections and continuous i.p. infusions could not penetrate the tumour cap. In contrast, free DNAzymes as well as PEG-polyplexes injected through the intravascular route did not enter the tumour area and remained extracellular, respectively. Thus, this showed that although transferrin did not affect the biodistribution of DNAzymes in mice, transferrin targeting was required for uptake and retention by tumour cells.

In conclusion, this seminal study demonstrated the suitability of transferrin-polyplexes as DNAzyme delivery systems for systemic administration, with the advantages of longer tumour retention of DNAzymes and more efficient tumour cell targeting [35]. As a potential model for DNAzyme delivery, the authors have shown the efficiency of cyclodextrin-based polymers in encapsulating DNAzymes and delivering them intravenously to tumour sites and into cancer cells. However, there were no attempts to demonstrate the effect of polyplexes on the tumours in terms of tumour response or regression and toxicity or side effects from the DNAzymes or delivery system. It would be desirable to attain toxicity profiles of these polyplexes due to the usage of a variety of

Table 2. *In vivo* testing of DNAzymes in animal models representing two major pathologies.

Disease	mRNA target	Animal	Xenograft	Delivery mode	Dose regimen	Anticancer activity	Ref.
Cancer							
			T79		Commencement of experiment, 50 µg of Dz co-mixed with 2.5 µL Fugene-6 reagent injected subcutaneously	90% reduction in tumour size	[10]
	c-Jun	Mouse	B16	Co-mixed with cell inoculation	Commencement of experiment, 750 µg of Dz co-mixed with 2.5 µL Fugene-6 reagent injected subcutaneously	60% reduction in tumour size, reduction in blood vessel density	[9]
			SaOS-2		Commencement of experiment, 250 ng of Dz co-mixed with 2.5 µL Fugene-6 reagent injected orthotopically into proximal tibiae	90% reduction in tumour size, preservation of growth plate cartilage and surrounding soft tissue	[23]
	VEGFR-2	Mouse	MDA-MB-435	Intratumoral	Four injections of 30 µL, each of 2.9 µg of DNAzyme and 1.092nmol his-lys polymer	75% reduction in tumour size, cell death in tumour periphery, reduction in blood vessel density	[8]
	Egr-1	Mouse	MCF-7	Intratumoral	Twice a week injections containing 20mg of DNAzyme and 1mL Fugene-6.	80% reduction in tumour size, reduction in blood vessel density	[24]
			MDA-MB-231		When tumours were palpable, single injection of 10 µL, of 10 µg of free DNAzyme	67% reduction in tumour size	[25]
	Co-therapy of uPAR DNAzymes and rPEDF	Mouse	SaOS-2	Co-mixed with cell inoculation	Commencement of experiment, 0.4 µM of Dz co-mixed with 125 nM rPEDF prior to orthotopic injection into proximal tibiae	50% reduction in primary tumour growth, pulmonary metastasis inhibition, decreased osteolysis	[26]

Table 2. *In vivo* testing of DNAzymes in animal models representing two major pathologies (continued).

Disease	mRNA target	Animal	Delivery mode	Dose regimen	Effect	Ref.
CVD	*Egr-1*	Rat and pig balloon catheter injury	Dz-gel mix applied around injured vessel	500 µg Dz co-mixed with Superfect transfection agent in presence of 1mM $MgCl_2$ and P127 Pluronic gel	Inhibition of neointima formation	[27-29]
		Rat myocardial ischaemia–reperfusion injury	Intramyocardial	500 µg Dz co-mixed with 60 µL Fugene-6 reagent and 1mM $MgCl_2$	Attenuation of infarct size and inflammatory mediators	[30]
	c-Jun	Rat carotid artery ligation	Dz-gel mix applied around injured vessel	750 µg Dz co-mixed with 30 µL Fugene-6 reagent, 1mM $MgCl_2$ and P127 Pluronic gel	Inhibition of neointima formation	[10]
	TNF-α	Rat acute myocardial infarction (AMI) by LAD ligation	Osmotic minipumps, intraperitoneal	1 µg /kg/h Dz co-mixed with DOTAP liposomes	Increased cardiac output	[31]
	VDUP1	Rat AMI by LAD ligation	Intracardiac	500, 200 or 10 µM Dz	Decreased apoptosis and collagen expression, increased cardiac function	[32]
	PAI-1	Rat AMI by LAD ligation	Intramyocardial	300 µg Dz co-mixed with 20 µL Superfect	Enhanced neovascularisation, cardiomyocyte regeneration and function recovery with angioblast co-injection	[33]

chemicals in their formulation and also the usage of a transferrin targeting molecule.

Transferrin is an iron-carrying protein whose receptor is highly expressed in rapidly growing cells, especially cancer cells, in an effort to process more iron, and has been used to target plasmids for tumour delivery [36]. In this case, transferrin might aid in targeting DNAzymes to blood-rich organs (such as the kidney, liver, heart and lungs) – as has already seen to the kidney and lungs in this report – and inducing possible side effects in these organs, in addition to the tumours. It is unfortunate, however, that no further reports were published regarding this DNAzyme delivery system.

Dendrimers, synthetic macromolecules with a well-defined, highly branched molecular structure, have been introduced as gene transfection or delivery systems. In the work by Tack *et al.*, commercially available poly(propylene imine) (PPI) dendrimers were modified through a series of complex conversion steps: i) at the exterior primary amines with acetyl groups or glycol gallate (PEG-like) groups; and ii) at the interior tertiary amines with methyl iodide (MeI) or methyl chloride (MeCl) to produce multiple quaternised cationic sites in the core of the dendrimer [37]. The authors then aimed to characterise these dendrimers for their DNAzyme-binding capacity, toxicity, *in vitro* transfection efficiency and *in vivo* delivery characteristics. Modified dendrimers termed G4 (MeI), bearing more cationic sites per molecule than the G2 (MeI) dendrimer, bound DNAzymes more efficiently. A comparison between acetylated and pegylated G4 dendrimers also showed that acetylated dendrimers bound DNAzymes better, with the advantage of a reversible binding that enables the dissociation of the complex and facilitates DNAzyme release.

Subsequent *in vitro* tests showed that the dendrimers displayed high transfection efficiency, especially with acetylated quaternised dendrimers G4 (MeI) and G4 (MeCl) displaying the best results of > 80% transfection efficiency, into ovarian carcinoma A2780 cells. This could be achieved at lower charge ratios of DNAzyme : dendrimer as compared to other transfection reagents, which require higher concentrations or higher charge ratios. A comparison with commercial cationic liposomal transfection agent DOTAP® (Roche Applied Science, Australia) showed comparable transfection efficiencies with the dendrimers; however the toxicity of DOTAP became high when DOTAP was used at similar concentrations as the dendrimers. Previous studies have also reported the ability of DOTAP to induce inflammatory response elements such as TNFα, IL-12 and IFN-γ in an *in vivo* setting [38], hence possibly contributing to the numerous *in vivo* mortalities. It was also established that free DNAzymes without dendrimers as delivery systems or transfection agents only transfected cells with an efficiency of about 5 – 10%. Toxicity tests to access the suitability of these dendrimers as gene transfection agents were conducted using the cytotoxicity MTT assay, and it showed that most of the dendrimers had low levels of toxicity, although G4-PEG (MeI) became toxic at concentrations above 10 μM. Unfortunately, this was the dendrimer eventually chosen for *in vivo* delivery of DNAzymes, as the other less toxic dendrimers produced insoluble precipitates when formulated in large concentrations for *in vivo* delivery. Dendrimer–DNAzyme complexes were fluorescein isothiocyanate (FITC)-labelled prior to intravascular injection in ovarian carcinoma-bearing mice. Whole body imaging then revealed a weak externally visible FITC fluorescence near the tumour which was subsequently shown to accumulate in the tumour and within the cancer cell nucleus.

In conclusion, the reported dendrimer system for DNAzyme delivery has been proven as a potential method for efficiently encapsulating DNAzymes and transfecting them into cells without significant toxicity, as well as accumulating in the tumour [37]. Despite the optimism, there are some concerns regarding this report. First, although the authors reported transfection of DNAzymes into ovarian carcinoma cells, the authors failed to examine the effects of the DNAzymes on the cells or their toxicity to other non-cancerous cells. As DNAzymes are catalytic constructs capable of downregulating target mRNA, it is imperative to examine this effect and its resultant consequences on the ovarian carcinoma cells. Thus, the question remains whether delivered DNAzymes would still retain their activity and be able to produce a therapeutic effect on target cells. This leads to the second point, regarding the ambiguity of the utilised DNAzymes, since a random DNAzyme sequence was generated and used in the experiments. The question regarding the effects when dendrimers delivered the DNAzymes into target cancer cells and possibly also normal cells remains unanswered. Thirdly, as the authors have also mentioned, *in vivo* toxicity would have to be further assessed by examining if DNAzymes were delivered to other organs of the mice by dendrimers. Thus, with further clarification of these issues, the real potential and substantial biological output of PPI–dendrimers could be further gauged. However, no further reports were made about these dendrimers and the authors have instead moved on to DNAzyme delivery using gold nanoparticles [39].

These gold nanoparticles were prepared by the reduction of tetrachloroauric [III] acid with sodium citrate [39]. The authors aimed to produce and characterise DNAzyme delivery particles that could effectively encapsulate DNAzymes and deliver them into HT29 human colon carcinoma cells. The resultant gold nanoparticles ranged from 1 to 90 nm in diameter, however only particles of 10 nm were chosen for subsequent analyses. Initial screening experiments suggested little uptake of the gold nanoparticles or the DNAzyme–gold nanoparticles into cells, thus targetors (molecules which aid in directing delivery systems to target) were added to facilitate interaction and internalisation of the nanoparticles via receptor-mediated endocytosis or other related processes.

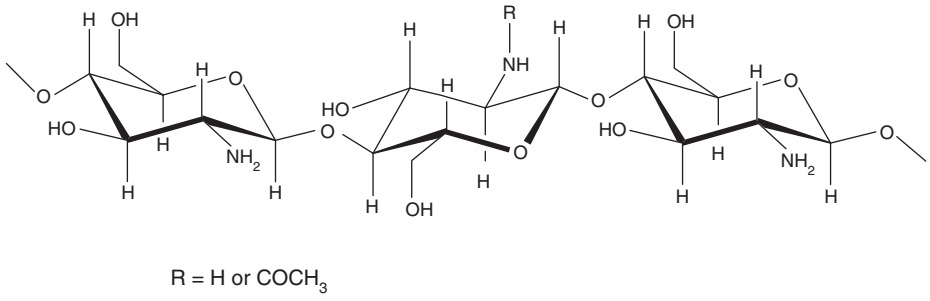

Figure 3. Chemical structure of chitosan polysaccharide.

The chosen targetors were conjugates between polylysine (PL) or polyethylenimine (PEI) and transferrin (Tf). In some formulations, polyvinyl pyrrolidone (PVP) was also added as a co-stabiliser. The optimal binding of the PL-Tf or PEI-Tf conjugate to DNAzyme was then examined by analysing various charge ratios of the two components on agarose gel and then looking for migration in an electric field.

Experimental data showed that PL-based nanoparticles were less efficient at cell transfection with DNAzymes as compared to PEI-based nanoparticles and also more toxic to HT29 cells. The most efficient transfections were obtained with PEI-based gold nanoparticles loaded with DNAzymes at low pH, low DNAzyme concentrations and in the absence of the PVP stabiliser. The authors also reported that this PEI-based formulation offered the best DNAzyme delivery rates of 56% (total transfection) and only 36% if only live cells were counted. It is, however, unclear if the 20% decrease in cell viability was due to the toxicity of the gold nanoparticles or the activity of the *c-myc* DNAzymes, as the author did not further clarify the results. Despite the lack of conclusive evidence as an effective DNAzyme delivery system, the authors pushed for the possibility of colloidal gold-based delivery of DNAzyme through the use of cellular targetors such as transferrin. Once again, the results from these experiments failed to distinguish between delivering DNAzymes into cells and observing appropriate biological outcomes from the delivery of these DNAzymes. Thus, these results are insufficient to determine whether the delivery of DNAzymes into cells was indeed successful in retaining the activity of DNAzymes to cleave target mRNA and act as potential gene therapy constructs.

Such concerns were partially addressed when *c-Jun*-targeted DNAzymes encapsulated into chitosan nanoparticles were tested for activity against osteosarcoma [40]. The DNAzymes, designed against the *c-Jun* proto-oncogene, which is over-expressed in osteosarcoma [41,42], downregulated *c-Jun* causing cell death [10], but more specifically cell death mediated through apoptosis [23]. Although initial results proved encouraging, the authors believed that the use of a DNAzyme delivery system would further enhance the applicability of the DNAzymes. Thus, initial studies were conducted through the encapsulation of *c-Jun* DNAzymes, Dz13, within novel cationic multilamellar vesicle liposomes [26]. These DDAB : DOPE liposomes were found to proffer better cell uptake of DNAzymes as compared to the Fugene-6 commercial transfection reagent. The increased cell uptake of DNAzymes was reflected in the slightly better rate of apoptosis induction in osteosarcoma 143B cells. Combining liposomal-Dz13 with 143B cells prior to orthotopic implantation into the tibia of mice resulted in significant inhibition of primary tumour growth, as well as decreased metastases in the lungs. Accompanying these *in vivo* results, histological analyses also confirmed less bone destruction at the site of the lesion of liposomal-Dz13 treated mice, confirming less tumour progression at the primary tumour site. A downfall of this study lies in the lack of a toxicity study. Although the authors had previously established that Dz13 was not toxic against normal cells resident in the bone lumen (human bone marrow mononuclear cell; HBMMNC) and did not inhibit their proliferation, the authors failed to establish the safety or side effects of the liposomal delivery systems. Thus, based on the reported efficacy demonstrated in this study, the authors proceeded to examine a biocompatible polysaccharide, chitosan (**Figure 3**), as a potential DNAzyme delivery system [40].

Chitosan has been described as a biocompatible polysaccharide which serves as a useful biodegradable sustained release depot [43]. In addition, it is readily available through the exoskeleton of crustaceans and is considerably cheaper than other vehicles such as liposomes or synthetic particles that involve biohazardous procedures.

In this study, Dz13 was encapsulated via a simple complex coercavation method to yield Dz13–chitosan nanoparticles with a median diameter of 350 nm, a high positive surface charge and high encapsulation efficiency (**Figure 4**). Apoptosis-mediated cell death of Dz13 was enhanced through the encapsulation and delivery by chitosan nanoparticles in osteosarcoma SaOS-2 cells. The particles were efficient at intracellular delivery as determined through scanning electron microscopy (SEM), showing the internalisation of the particles into endosome-resembling vesicles within 48 h. These Dz13–chitosan nanoparticles were stable at room temperature for up to a month and in serum for seven days

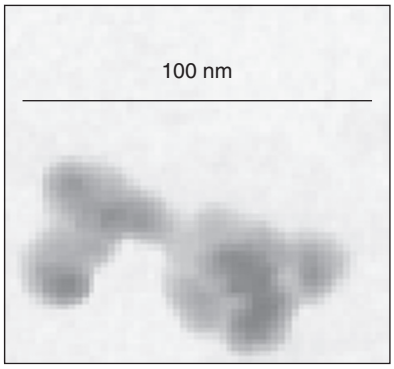

Figure 4. Scanning electron microscopy (SEM) image of biocompatible chitosan–DNAzyme nanoparticle. Chitosan–DNAzyme nanoparticle observed as small (grape-like) clusters. Chitosan–DNAzyme nanoparticles typically have a ζ-potential of +14 mV, encapsulation efficiency of 94% and loading rate of 4.7%.

without any significant loss in activity. Subsequent *in vivo* tissue analysis following intratibial and intramuscular injection of the particles revealed no local tissue reactivity, ruling out any possible toxic side effects to surrounding structures. Radiography and histology also demonstrated patent bone structure and undisturbed muscular morphology in mice administered intratibial or intramuscular Dz13 nanoparticles. In addition, only a low concentration of 500 ng Dz13 was required for *in vivo* applications, as compared to the mg/kg doses required for a similar RNA construct – siRNA – to be potent [44].

4. Expert opinion

The field of DNAzyme technology has evolved in the past decade from being a mere item of curiosity possessed with cleavage capacity, to an actual potential drug agent. As this review highlights, in two different disease indications, cancer and cardiovascular disease, this class of molecules has shown the potential to be efficacious. However, current approaches still rely on sub-optimal drug delivery systems (DDSs) for DNAzymes. There is a clear need for DDS research groups to play catch-up so that better DDSs, which can be adopted clinically in future, are developed for DNAzymes. This is now imperative given the anticipative imminence of the first clinical trial for DNAzymes. Certain DDSs have shown potential, such as chitosan and PEI, although more emphasis needs to be placed on actual efficacy and safety, as well as on the pharmacokinetics of the molecule being delivered. Unfortunately, the plethora of DDSs reported for antisense delivery – the trailblazer for target gene knockdown agents – have not yielded even one entity capable of being used clinically, and clinicians have resorted to administering continuous systemic free oligonucleotides with promising, albeit lukewarm, results. The challenge for DNAzymes to be considered a genuine drug candidate alongside siRNA and antisense really and simply lies in the better implementation of DDSs.

Declaration of Interest

The authors state no conflict of interest and no payment has been received in preparation of this manuscript.

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