The cellular delivery of antisense oligonucleotides and ribozymes

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The design and development of antisense oligonucleotides and ribozymes for the treatment of diseases arising from genetic abnormalities has become a real possibility over the past few years. Improvements in oligonucleotide chemistry have led to the synthesis of nucleic acids that are relatively stable in the biological milieu. However, advances in cellular targeting and intracellular delivery will probably lead to more widespread clinical applications. This review looks at recent advances in the *in vitro* and *in vivo* delivery of antisense oligodeoxynucleotides and ribozymes.

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▼ Antisense technology has become a real alternative in the treatment of disease states arising from genetic abnormalities such as gene amplification or over-expression. Antisense oligodeoxynucleotides (ODNs) duplexed with target mRNA can inhibit gene expression by several putative mechanisms, such as translation arrest mediated by the blockade of ribosomal read-through, and hydrolysis of mRNA by recruitment of RNaseH. By contrast, ribozymes inhibit gene expression by the direct hydrolysis of targeted mRNA. This is achieved because ribozymes contain sequence motifs, such as the hammerhead, that are able to perform transmolecular hydrolysis. Both ribozymes and antisense ODNs are undergoing evaluation in clinical trials as treatments for a variety of diseases, including cancer[1,2], viral infection[3,4] and inflammatory disorders such as Crohn's disease[5] (reviewed in Refs 6,7). Recently, the first antisense ODN drug, Fomivirsen (Vitravene™), developed by Isis Pharmaceuticals (Carlsbad, CA, USA), has received approval for marketing in the USA and Europe. Fomivirsen is administered by repeated injection into the vitreous of the eye and is used for the treatment of *Cytomegalovirus retinitis* infections in AIDS patients.

Antisense ODNs and ribozymes are macromolecules that are often polyanionic. As a result, a major pharmaceutical concern is the effective delivery of these molecules *in vitro* and *in vivo*. Many different strategies have been attempted for the improved delivery of ODNs and ribozymes with varying degrees of success. However, the aim of this review is not to be exhaustive in covering every strategy reported, but to give an overview of the most promising delivery systems for antisense ODNs and ribozymes that are currently available.

Design

Before addressing the delivery of antisense ODNs or ribozymes, it is first necessary to have a design that is active. The design of antisense ODNs and ribozymes appears straightforward at first, because the exact sequence of the target mRNA is known. The use of computer programs that predict RNA folding, such as MFOLD[8], should allow the identification of hybridization-accessible sites (i.e. single-stranded regions). This approach, however, has proved unsuccessful[9] and it is clear that currently available computer algorithms do not accurately predict the complex secondary and tertiary structure of RNA molecules.

Previously, a strategy of 'gene-walking' has been employed, whereby a series of ODNs has been generated against the target mRNA and each one evaluated in cell culture or *in vivo*. This strategy has been successful in identifying active sequences[10] but is far from exhaustive. To obtain a complete picture of RNA accessibility using this method would be time consuming and expensive. For example, as outlined in a recent review[11], identifying the most effective 20-mer antisense ODN against

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a 2 kb mRNA molecule would require the synthesis and testing of 1981 individual ODNs. Recently, two new strategies, RNaseH mapping and scanning combinatorial oligonucleotide arrays, have been adopted and both have the advantage of being far more exhaustive[11,12].

RNaseH mapping

RNaseH mapping relies on the fact that RNaseH is able to recognize and selectively degrade the RNA strand in an RNA–DNA heteroduplex. Combining the target mRNA with a random library of ODNs in the presence of RNaseH and analysing the cleavage fragments reveals accessible sites. This strategy has been used successfully to select potent antisense molecules against the human multidrug resistance (MRP1) gene[13], the RNA of hepatitis C virus[14] and active ribozyme constructs[15,16]. However, there is potential for multiple RNaseH cleavage sites within each DNA–RNA heteroduplex[17], which could lead to the imprecise selection of ODN sequences.

ODN scanning arrays

This novel strategy involves the use of scanning combinatorial ODN arrays for the determination of accessible sites within targeted RNA molecules[11,18,19]. Using a polymer (or glass) support and standard phosphoramidite chemistry, it is possible to synthesize an array of ODNs covering every possible site within the target RNA. A radiolabelled version of the target RNA can then be hybridized to the array, which elucidates suitable antisense sequences. This approach is exhaustive and can be used to cover the full length RNA; however, in practice this is tedious and often unnecessary, and so only short regions are used. It is possible to combine RNaseH mapping and ODN scanning arrays by using the former to select a region with good accessibility, followed by the latter to precisely identify active antisense sequences[20].

Stability

The lack of biological stability is a major problem in the use of DNA and RNA oligonucleotides (ONs) as therapeutic agents. Unmodified phosphodiester (PO) backbone ODNs are rapidly degraded in biological fluids[21,22] by a combination of both endo- and exo-nucleases. To overcome this, a variety of chemically modified ODNs have been developed that are significantly more stable[23,24]. The most widely studied of these are the phosphorothioate (PS) ODNs in which one of the non-bridging oxygens of the phosphodiester backbone is replaced with sulfur. More recently, other second-generation modifications, such as peptide nucleic acids (PNAs)[25,26], 2′-methoxyethyl[27,28] and morpholino-based ODNs[29,30] have also been investigated (Fig. 1a).

Similar to DNA, unmodified RNA is also rapidly degraded in the biological surroundings. For example, the half-life of an all-RNA hammerhead ribozyme in human serum is <6 sec (Ref. 31). Thus, various chem ical modifications have been applied in an effort to increase ribozyme stability while retaining catalytic activity, including the use of 2′-*O*-methyl ribonucleotides[32–34], chimeric RNA–phosphorothioate DNA[35,36] and 3′-3′-inverted thymidines at the 3′ termini[33,37] (Fig. 1b). However, ribozymes cannot be wholly modified, because some ribonucleotides are essential for catalytic activity. In hammerhead ribozymes, for example, the residues G5, A6, G8, G12 and A15 (numbered according to Ref. 38) have to remain as unmodified ribonucleotides to preserve catalytic activity.

Cellular uptake

Cellular uptake refers to the combination of both ODN membrane-binding and internalization. In cultured cells, the internalization of naked ODNs is generally inefficient, with only a few ODN molecules actually gaining entry to the cell[39]. Exogenously administered ODNs enter cells *in vitro* by a combination of fluid-phase (pinocytosis), adsorptive and receptor-mediated endocytosis[40]. However, this results in a trafficking problem because not all of the internalized ODN will be able to exert an effect by interacting with its intended subcellular targets. This is because the majority of internalized ODN is sequestered into endosome or lysosomal compartments, as characterized by the punctate cytoplasmic distribution and intracellular release of fluorescently labelled ODN after treatment with lysosomatropic agents[41]. A significant amount of the ODN is also compartmentalized within other cellular organelles, such as the Golgi complex and the endoplasmic reticulum[42]. A similar cellular distribution is observed for exogenously administered hammerhead ribozymes[37]. In both cases, little or no fluorescently labelled ODN is observed ‘free’ in the cytoplasm or nucleus, which are the sites of action for both ODNs and ribozymes. Microinjection of ODN into the cytoplasm of cells, bypassing the endocytic pathway, leads to a rapid accumulation of the ODN within the nucleus[43], which suggests that, after endosomal exit, ODNs are able to migrate to the nucleus. This free mobility of ODNs within the cytosol appears to be a significant advantage of antisense molecules over gene therapy, where the cytosol–nuclear migration of large DNA constructs might be a rate-limiting step to efficacy.

A recent review[44] highlighted the apparent conflict between *in vitro* and *in vivo* studies, with regard to the necessity of a delivery system. There are several examples in the literature where antisense effects have been achieved *in vivo* without the use of a delivery system[45,46]. However,

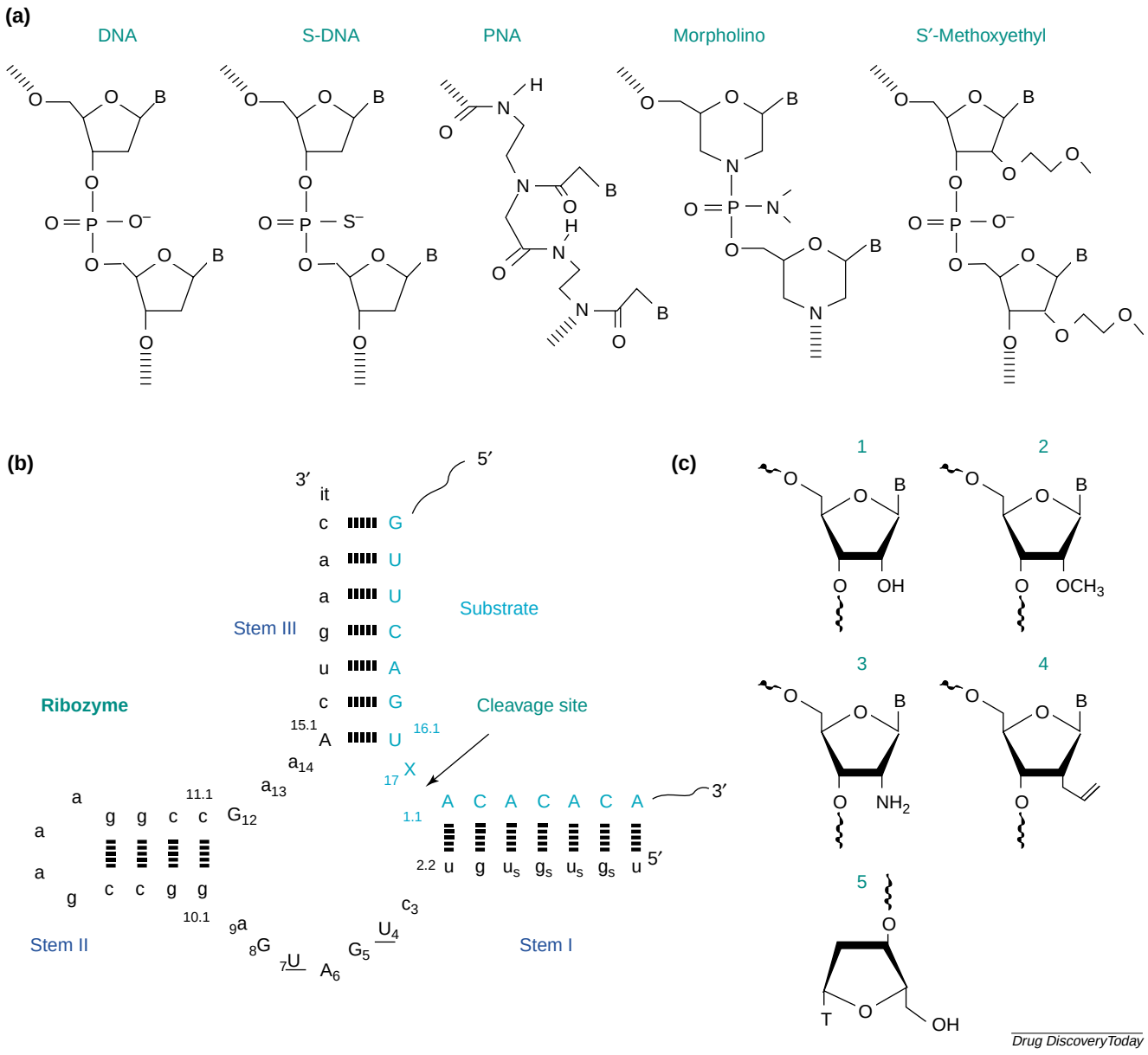

Figure 1. Commonly used nucleic acid chemistries. **(a)** depicts phosphodiester DNA chemistry and various standard chemical modifications aimed at enhancing the stability of ODNs. Abbreviations: S-DNA, phosphothiorate DNA; PNA, peptide nucleic acid. **(b)** A 'hammerhead' ribozyme**:** upper-case letters represent unmodified RNA; lower-case letters represent 2′*O*-methyl ribonucleotides; underlined letters represent either 2′-amino or 2′-*C*-allyl ribonucleotides. 3′-3′-inverted thymidine is represented by 'it'. **(c)** shows the structures of various forms of modified RNA: **1**, unmodified RNA; **2**, 2′*O*-methyl RNA; **3**, 2′-amino RNA; **4**, 2′-*C*-allyl RNA; **5**, 3′-3′-inverted thymidine.

these differences might relate to the contrasting ways in which the experiments are performed. For example, with *in vitro* studies, the effects of ODNs are usually assessed after the treatment of cells for a period of only a few hours. By contrast, animal experiments involve repeated administration, usually by multiple injections, resulting in prolonged exposure to the ODN. Despite the effectiveness of *in vivo* studies with free-ODNs, this does not obviate the need for developing delivery systems; such systems will probably lead to an improved efficacy of ODNs by reducing the amount and frequency of administration.

Enhancing ODN uptake and delivery

The aims for optimal delivery of ODNs and ribozymes are, therefore, enhanced cellular uptake, improved exit from subcellular compartments and correct targeting (spatial and temporal) to the desired site of action (Fig. 2). Successful targeting will require effective target-matched

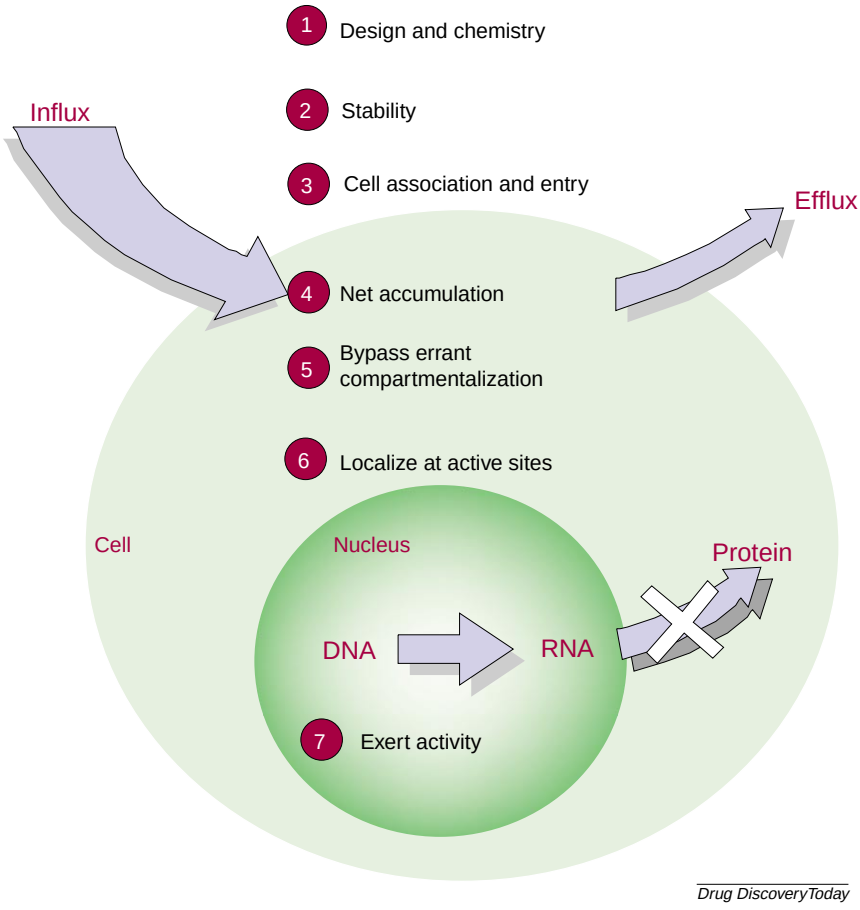

Figure 2. Requirements for the cellular delivery and activity of an antisense molecule. For an oligonucleotide (or ribozyme) to be efficacious it must: heteroduplex with the hybridization-accessible sites within the targeted mRNA and have the appropriate chemistry for effective hybridization (**1**); confer sufficient extra- and intra-cellular biological stability (**2**); have efficient uptake, usually involving cell association and entry (**3**); have net accumulation once inside the cell, where influx is greater than efflux (**4**); by-pass errant compartmentalization and remain bioavailable (**5**); localize at sites of bioactivity within the cell, such as the cytoplasm and nucleus (**6**); and exert its activity once inside the cytoplasm or nucleus, in the intended sequence-specific manner (**7**). Reproduced, with permission, from Ref. 7.

delivery and pharmacodynamics, in which effective concentrations of ODN at the target site will need to be maintained for an appropriate length of time. The required time-frame needs to be matched to the biological half-life of the target protein and to the desired level of knock-down. For example, repeated or sustained delivery for a time equivalent to the half-life of target will theoretically only result in 50% of the maximum potential effect. A delivery time period of ~four half-lives would be required to achieve >90% inhibition.

This concept is further discussed in the context of biodegradable polymers later in this review. Most of the strategies discussed in the remainder of this review have been tested in *in vitro* systems and, where not directly inferred, would be applicable to the delivery of both ribozymes and ODNs.

Lipid delivery systems

Liposomes and lipoplexes

Liposomes are composed of phospholipids arranged in a bilayer, which can either encapsulate nucleic acids within an aqueous centre, or can form lipid–nucleic acid complexes as a result of opposing electrostatic charges[47]. Liposomes are the most commonly used system for the *in vitro* delivery of nucleic acids, and afford protection to the nucleic acid while enhancing its cellular delivery. Although anionic and neutral liposomes have been studied for ODN delivery, their poor encapsulation efficiencies have prevented their widespread use[48,49].

Cationic liposomes and cationic lipid–nucleic acid complexes (lipoplexes) are the most successful delivery system for achieving biological effects in cell culture with both ODNs[50–52] and ribozymes[53,54]. By virtue of their positive charge, these delivery systems have a high affinity for most cell membranes, which are negatively charged under physiological conditions, and usually gain entry to cells by adsorptive endocytosis. To facilitate the subsequent release from endosomal or lysosomal compartments, many commercially available lipoplex transfection agents (also called transfectins or cytofectins) contain a helper lipid, such as dioelylphosphatidylethanolamine (DOPE). DOPE is an inverted-cone-shaped lipid that is thought to aid cytosolic release by fusing to and/or disrupting the endosomal membrane[55], possibly by the formation of hexagonal lipid structures[56]. Studies have also shown that ODNs can readily dissociate from lipoplexes in a bioavailable form within the cell.

Although the exact mechanism of function of the delivery system is unknown, it is clear that significant optimization of the charge ratio between the cationic lipid and the nucleic acid at a given dose is essential for effective delivery and activity. The effectiveness of lipoplexes is also dependent on the type and nature of the cationic lipid, cell type, ODN chemistry, ODN length and the method of complex formation. Recent studies have shown that only

cationic lipoplex formulations optimized for these variables will significantly improve the intracellular bioavailability and activity of ODNs[42,57]. Optimization of the amount of cationic lipid used is also essential to minimize the potential toxicity of the lipid to cultured cells[58]. In some biological systems, toxicity of a cationic lipid–nucleic acid complex might even mimic the nucleic acid activity[59].

Liposomes in vivo

In vivo studies with liposomes and lipoplexes[60] have shown that they are rapidly cleared from the circulation by the reticuloendothelial system (RES) when administered systemically[60–62]. Modification of liposomes with polyethylene glycol (PEG), to reduce opsonization and phagocytosis by cells of the RES, resulted in sustained circulation of ODN–liposomes[63]. Such systems might allow the enhanced passive-targeting of tumours, which have 'leaky' vasculature that is permeable to small-sized liposomes.

In a study by Kisich and colleagues, cationic lipids were used to deliver synthesized ribozymes to peritoneal macrophages *in vivo*[64]. Three different lipids were compared: *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethyl ammonium chloride (DOTMA), 2,3-dioleyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and *N*-(1-(2,3-dimyristyloxypropyl)-*N*,*N*-dimethyl-(2-hydroxyethyl) ammonium bromide (DMRIE). The macrophages incorporated tenfold more ribozyme when delivered in conjunction with DOSPA than with the other cationic lipids. More recently, the same group reported specific inhibition of tumour necrosis factor-α (TNF-α) expression *in vivo* following ribozyme treatment[65]. Following intraperitoneal injection of cationic lipid–ribozyme complexes, ~6% of the administered ribozymes as accumulated by target macrophages. The catalytically active ribozymes were found to suppress TNF-α secretion by 50%, relative to the inactive control.

Despite the few successful uses of cationic lipids *in vivo*, a major pharmaceutical hurdle that remains is the development of a non-toxic, serum-stable, lipoplex formulation that can be routinely used to achieve reproducible biological effects with ODNs and ribozymes.

pH-sensitive liposomes

Another development, aimed at enhancing release from endosomal compartments, is the use of pH-sensitive fusogenic liposomes. These consist of a non-bilayer-forming lipid, such as DOPE, and a titratable acidic amphiphile such as oleic acid (OA) or cholesterylhemisuccinate (CHEMS)[66]. At pH 7 the amphiphile maintains the lipid mix in a bilayer (liposome) structure; however, as the complex moves through the endosomes the pH decreases and the amphiphile becomes protonated. This causes the liposome to collapse, resulting in fusion with the endosomal membrane and release of the liposome contents into the cytoplasm. However, the anionic nature of pH-sensitive liposomes could lead to poor encapsulation of ODNs.

Cationic amphiphiles

Recently, a novel group of cationic amphiphiles has been discovered[67]. These molecules consist of a hydrophobic cholic acid group, covalently linked to spermine and/or spermidine groups, which enables association with nucleic acids. One of these compounds was able to efficiently deliver ODNs, resulting in a 250-fold increase in cellular uptake compared with naked ODNs[67]. Unlike other cationic lipids, this compound is also active in the presence of high concentrations of serum and does not require a neutral lipid to facilitate ODN delivery to cells.

Dendrimers

Dendrimers comprise a relatively new macromolecular delivery system and possess a highly branched three-dimensional (3D) structure. They are attractive as a delivery vehicle, because they are synthesized by defined polymerization reactions yielding a monodisperse globular product with a large amount of controllable peripheral groups. Several categories of dendrimers have been synthesized, with various functional groups, depending on the initial monomer and the building block monomers used. To date, most studies regarding dendrimer delivery of ODNs have been performed using the polyamidoamine (PAMAM) starburst dendrimers[68–70] (Fig. 3). PAMAM starburst dendrimers possess a hydrocarbon core, charged surface amino groups, and have a well-controlled chemistry. The cationic surface groups facilitate the binding of anionic ODNs.

Stable complexes between ODNs and cationic dendrimers form under a variety of conditions, such as water, phosphate-buffered saline and serum[68]. PAMAM dendrimers mediate the high efficiency transfection of several cultured mammalian cell lines[70]. Dendrimer–ODN complexes cause a 75% reduction in metabolic degradation of phosphodiester ODNs in serum, and also afford protection from the lysosome[71]. A recent study[72] examined the delivery of ODNs with PAMAM dendrimers, and found that the dendrimer remains complexed with the ODN as it enters the cell nucleus. Interestingly, it was also reported that conjugation of the dendrimer to a small fluorescent dye enhanced its ability to deliver ODNs and increased its effectiveness in serum. This offers the potential for tailoring the dendrimer surface structure to achieve the desired uptake properties.

The reproducible and defined small size of dendrimers offers a significant advantage over other particulate systems.

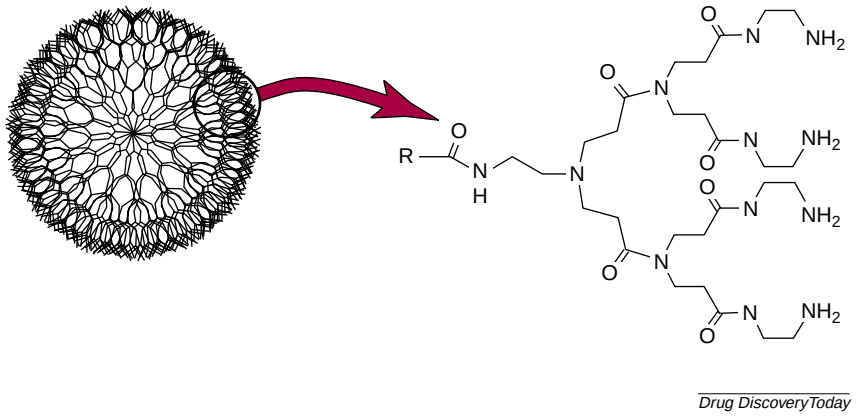

Figure 3. Representation of the globular structure of the PAMAM dendrimer. Enlargement shows the regular chemical structure and the terminal amino groups that facilitate nucleic acid binding.

However, extensive testing is necessary to establish biocompatibility and to fully evaluate the potential use of dendrimers for delivering antisense molecules.

Biodegradable polymers

The relatively rapid degradation of many nucleic acids in the biological surroundings *in vitro* and *in vivo* suggests that the pharmacological effects of ODNs are likely to be short-lived. Furthermore, the duration of action of ODNs might also comprise a rapid redistribution and pharmacokinetic elimination half-life (even chemically stabilized PS-ODNs are cleared rapidly, with a plasma half-life of 1 h[73]). Thus, repeated administration of ODNs is often required for efficacy[6]. This would be particularly important for targets with long half-lives. For example, in the case of peripherin, a neurone-specific intermediate-filament protein that has a slow turnover (half-life of ~7 days), repeated administration of antisense ODNs for up to 40 days was required for a 90% reduction of peripherin levels in cultured PC12 phaeochromocytoma cells[74].

The use of biodegradable polymeric delivery systems, which provide sustained release of the active compound, could obviate the need for repeated administration by improving both the pharmacokinetics and the pharmacodynamics of ODNs and ribozymes. The entrapment of ODNs within such polymeric matrix systems could improve ODN stability, reduce the dose of ODN required for efficacy, and reduce toxicity or non-specific activities associated with ODNs.

The most widely studied polymers are polylactides (PLA) and co-polymers of lactic acid and glycolic acid P(LA–GA), and these have been evaluated for their potential as delivery agents for antisense ODNs[75–77] and ribozymes[34]. These thermoplastic polyesters can be fabricated in any shape as implantable devices either for local delivery, or as parenterally administered systemic formulations. Indeed, they are used routinely in resorbable surgical sutures[78] and in commercially available sustained-release preparations[79,80] (e.g. Zoladex™; AstraZeneca, Alderley Edge, UK).

Lewis and coworkers[77] reported on the biological stability, hybridization potential and *in vitro* release kinetics of antisense PO- and PS-ODNs entrapped within biodegradable PLA film (100 ± 10 μm thickness) matrices. Sustained release of ODNs was observed from these devices for >1 month in physiological buffer solutions, and was dependent on ODN chemistry (PS-ODNs were released more slowly than PO-ODNs) and on ODN length (a 20-mer was released more slowly than a 7-mer). The polymer-entrapped ODNs were protected from serum nucleases and were thought to retain their biological activity for the entire study period. Gel mobility shift analyses and duplex melting-point determinations suggested that the hybridization capability of antisense ODNs released from the PLA matrices was unaffected by the solvent-casting procedure used for preparing these polymer devices. Subsequently, it was reported that these device fabrication procedures also had no effect on the biological activity of ribozymes[34], of which the 3D structural conformation is deemed crucial for catalytic activity[81].

More recently, studies with P(LA–GA) microsphere devices for entrapping ODNs have shown similar advantages with using biodegradable polymer delivery systems[75,82]. Lewis and colleagues[75] prepared P(LA–GA) microspheres containing ODNs using a double-emulsion method and evaluated the release of the encapsulated ODNs. They showed that release-profiles could be controlled, and ultimately tailored to specific requirements, by altering the size of the microspheres, the amount of ODN loading and the length of the ODN[75] (Fig. 4). In several cases, the *in vitro* release-profiles of antisense ODNs from P(LA–GA) matrices were shown to be triphasic[76,77]: typically, profiles were characterized by an initial 'burst effect' during the first 48 h (phase 1) of release, followed by a more sustained release (phase 2) with a subsequent additional release (phase 3) resulting from bulk degradation of the microspheres (Fig. 4b).

Cellular delivery of ODNs using polymers

Polymeric microsphere (or even nanosphere) devices could potentially enhance the delivery of ODNs at the cellular

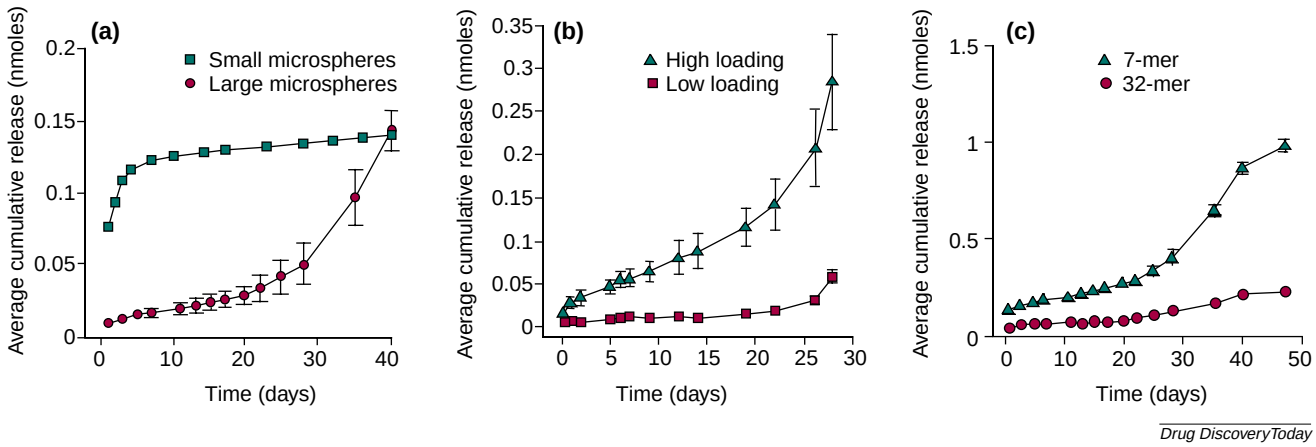

Figure 4. Factors controlling ODN release from biodegradable polymer microspheres. **(a)** Particle size: the average cumulative release in nanomoles (nmoles), over 40 days, of 20-mer phosphodiester (PO) ODN from lactic acid and glycolic acid co-polymer [P(LA–GA); MW = 3 kDa] microspheres in phosphate-buffered saline (PBS) at 37°C. Small spheres = 1–2 μm and large spheres = 10–20 μm. Loading = 50 ng ODN per mg of polymer. **(b)** ODN loading: fixed microsphere size was 10–20 μm. High loading = 300 ng ODN per mg of polymer, low loading = 50 ng ODN per milligram of polymer. **(c)** ODN length: average cumulative release (nmoles) of 32-mer and 7-mer PO ODNs, over 50 days, from P(LA–GA) microspheres (10–20 μm; MW = 3 kDa) in PBS at 37°C. Loading = 110 ng ODN per mg polymer.

level because of their relatively small size. They could also provide site-specific delivery to a particular tissue or subset of cells and/or provide sustained delivery of the free-ODN into the systemic circulation following implantation (either intramuscularly, subcutaneously or intraperitoneally). In an attempt to evaluate the potential for delivery at the cellular level, Akhtar and Lewis[82] administered microspheres containing anti-HIV ODNs to cultured murine macrophages. They showed that P(LA–GA) microspheres in the 1–2 μm size-range improved the cellular delivery by up to tenfold compared with free-ODNs[82]. In the same study, uptake of ODNs was significantly reduced in the presence of metabolic and phagocytic inhibitors, indicating that the microspheres laden with ODNs were entering cells by an endocytic or phagocytic mechanism. However it is not clear, as yet, how the polymer-entrapped ODNs are trafficked through the cells, and how and when they are released from the delivery system; these issues require further study.

In vivo *studies with polymers*

The potential for site-specific and sustained delivery of ODN-loaded polymer microspheres to tumours and specific regions of the brain has also been examined[83]. Using fluorescently labelled nucleic acids, a single, local injection of naked ODNs within an A431 tumour xenograft in nude mice resulted in its rapid elimination from the tumour with only ~20% of the dose remaining after 6 h. By contrast, the use of ODN-loaded microspheres maintained ODN delivery for >48 h within the tumour. Khan and coworkers[83] stereotaxically administered ODNs into the neostriatum of rat brain, which further confirmed the sustained-release capability of these devices *in vivo*. Interestingly, these authors also found that ODNs delivered as polymer microspheres into brain tissue improved the subcellular biodistribution of ODNs that might enhance antisense activity. They confirmed that naked PS-ODNs are predominantly taken up by neuronal cells and ultimately localize within vesicular structures, as indicated by the punctate distribution of fluorescence in these cells. However, with slowly released PS-ODNs from the P(LA–GA) microsphere formulation, the biodistribution profile was characterized by a more diffuse cytosolic and nuclear fluorescence in neuronal cells. Furthermore, because a 20-fold-lower dose of ODNs in microspheres yielded an intensity of signal similar to that generated by free-ODNs, it was suggested that much lower doses of ODNs will be necessary for antisense activity if delivered as a biodegradable polymer formulation.

The improved efficacy of ODNs delivered as polymer formulations has been reported in a recent study by Putney and colleagues[75]. By using male CD-1 nude mice injected with human melanoma cells as an *in vivo* model, the study showed that the subcutaneous delivery of a P(LA–GA)-microsphere-encapsulated antisense-ODN, targeted against the oncogene *c-myc*, led to an enhanced suppression of tumour growth when compared with free-ODN administered intravenously. The delivery of ODN subcutaneously in microspheres (6 mg) resulted in ~60% inhibition of tumour growth, whereas the same dose of ODN in solution

(administered over 8 days) only achieved 20% inhibition. Western blot analysis also revealed a more prolonged reduction in c-myc protein levels (55% after 20 days administration) with microsphere-encapsulated ODN compared with free-ODN administered in solution (no reduction after 20 days).

Polymer nanoparticles

Biodegradable polyalkylcyanoacrylate (PACA) nanoparticles have been investigated for the delivery of nucleic acids. PACA nanoparticles are obtained by emulsion polymerization of various alkylcyanoacrylate monomers in acidic medium. Because of the negative surface charge of PACA nanoparticles, a cationic copolymer or cationic hydrophobic detergent is used to facilitate ODN binding. In contrast to P(LA–GA) microspheres, in which ODNs are physically entrapped within the polymer matrix, ODNs are adsorbed onto the charged surface of the PACA nanoparticles. This system has proved to be efficient, not only for protecting the ODN from degradation from exonucleases, but also for increasing the uptake of ODNs[84–86]. The uptake of PACA nanoparticles was shown to be temperature-dependent, suggesting an endocytic or phagocytic process, and the nanoparticles accumulated in lysosome or phagosome vesicles[86]. Schwab and colleagues[87] have demonstrated that an antisense ODN targeted against another oncogene, Ha-*ras*, is effective at a 100-fold-lower concentration when adsorbed onto PACA nanoparticles. However, the toxicity of the hydrophobic cations and the production of formaldehyde upon polymer degradation could limit the *in vivo* use of this system.

The use of sustained-release polymers provides a proven method of improving the pharmacokinetics and pharmacodynamics of ODN delivery. This is especially true for long-lived targets, which would require repeated administration of ODNs for efficacy *in vivo*. In these cases, careful selection of polymer formulation variables, such as polymer type, polymer MW, and the size and nature of the polymer device, might achieve tailored or target-matched sustained delivery. However, the widespread use of polymer microparticles and nanoparticles for delivering nucleic acids will require improvements in entrapment efficiencies and drug loadings. By virtue of their small size, the dose that can be safely delivered by these devices in a manageable volume for administration is limited. This is a significant hurdle that needs to be cirumvented and is, therefore, the subject of further studies.

Receptor-mediated endocytosis

Receptor-mediated endocytosis could potentially be used for the effective targeting of ODNs and ribozymes to subsets of cells or specific organs. Tumour cells demonstrate a propensity to this kind of targeted delivery because they have an increased requirement for essential nutrients compared with benign cells.

Using a chemically modified ribozyme, conjugated to a transferrin receptor antibody (TFA), Hudson and coworkers[88] were able to demonstrate a threefold increase in cellular uptake compared with free ribozyme. This uptake was temperature-dependent, indicative of a receptor-mediated process, and inhibited by excess free-TFA. Rojanasakul and colleagues[89] showed that by using polylysine conjugated to mannose they could efficiently deliver an antisense molecule directed against TNF-α by exploiting the macrophage-specific mannose receptor. At low ODN concentrations (25 μg ml^{-1}) a 17-fold increase in cellular uptake, compared with free-ODN, and >90% TNF-α inhibition was observed. Similarly, another study[90] used folate to target antisense ODNs to ovarian cancer cells overexpressing the folate receptor. The uptake of folate-conjugated ODN was eightfold greater than free-ODN and was inhibited by free-folate. Chauduri and colleagues[91] used liposomes coated with an artificial ligand, maleylated bovine serum albumin (MBSA), to target a 17-mer antisense ODN to the macrophage scavenger receptor. The ODN was complementary to the common 5′ end of every mRNA in *Leishmania mexicana amazonensis*, which resides in the phagolysosomes of cells it has infected. The MBSA–liposome-encapsulated antisense ODN was able to kill >90% of the parasites within 5 h, compared with 20% using free-ODN, and no detectable cell damage was observed.

In vivo *studies*

Several studies have looked at using the asialoylglycoprotein receptor (ASGP-R), which is unique to the liver, to target nucleic acids. A study directly linking a ligand for the ASGP-R to an ODN showed that the ligand–ODN is more effectively delivered to parenchymal liver cells *in vitro* compared with free-ODN[92]. Furthermore, injection of the ODN and ODN–ligand into male Wistar rats showed that the specific accumulation of ODN in parenchymal cells was improved almost 60-fold by the ligand. Kren and colleagues[93] used polyethyleneimine (PEI) conjugated with lactose to target a chimeric RNA–DNA ODN to ASGP-R. The chimeric ODN was directed to the rat factor IX gene and was designed to introduce a single point-mutation to inactivate factor IX. Injection of the free chimeric molecule into male Sprague-Dawley rats resulted in no detectable mutations, but when administered with the PEI–lactose conjugate a dose-related genomic DNA mutation was observed in up to 40% of animals.

Combined approaches

Although receptor-mediated delivery strategies facilitate the targeting to a specific cell-type or tissue and, depending on the abundance or affinity of the receptor target, can also increase the extent of ODN cellular association, their successful application will also require the correct intracellular distribution of the ODN in a bioavailable form. For this to occur, there should be minimal entry of the active ODN to inappropriate (or non-target) compartments, and the ODN must be active (hybridization-accessible) following the dissociation of the ODN from the receptor–delivery complex.

Combination approaches, such as the inclusion of endosome-disrupting agents and labile linkages between the targeting moiety and ODN, could have some value, providing that the resultant complexes and/or formulations do not become too large or cumbersome. For example, Skalko and colleagues[93] used an *N*-acetylglucosamine derivative of BSA, conjugated to a pH-sensitive liposome with FITC–dextran, as a mimic of antisense ODNs. An avian hepatoma cell line expressing ASGP-R efficiently took up this targeted liposome, compared with liposomes with no targeting ligand. A diffuse cellular fluorescence for the FITC–dextran was also observed, suggesting that the internalized FITC–dextran had escaped from endosomal compartments.

Carrier-peptide-mediated delivery

Protein transduction domains are small motifs in proteins of approximately 10–16 amino acids in length. They can be used as 'carrier' or 'transport' peptides to promote the delivery of active agents across biological membranes by a receptor- and transporter-independent mechanism. Several of these peptide motifs have now been identified, including the Tat protein from the HIV-1 virus, the *Drosophila melanogaster* homeotic transcription factor ANTP, and the herpes simplex virus type-1 (HSV-1) VP-22 transcription factor[95].

There are several examples in the literature of peptides being used for ODN delivery. For example, Astriab-Fisher and coworkers[96] used peptides derived from the Tat and Ant proteins conjugated to a PS-ODN to target the *MDR1* gene, which encodes P-glycoprotein. Both peptides were able to efficiently deliver the ODN to cells in culture. Surprisingly, the peptide–ODN conjugates inhibited P-glycoprotein expression more effectively in the presence of serum than in the absence of serum. Another group used a chimeric peptide (termed MPG) consisting of a hydrophobic fusion domain derived from HIV gp41 and a hydrophilic nuclear localization signal derived from the SV40 T-antigen[97]. They demonstrated that the MPG peptide was able to efficiently bind to both single-stranded and double-stranded ODNs (K_d = 1–2 × 10^{-8} M). The MPG–ODN complex improved the stability of the ODNs and no significant degradation was observed after 10 h in medium containing serum. Furthermore, this complex was efficiently delivered, with >90% of cells demonstrating uptake of fluorescently labelled MPG–ODN after 1 h. The cellular distribution of the MPG–ODN was predominantly nuclear, whereas free-ODN was restricted to subcellular vesicles, presumably endosomal. As previously, treatment at 4°C, which blocks endocytosis, had no effect on cellular uptake or distribution, suggesting a non-endocytic mechanism.

Although no reports exist on the use of these carrier or transport peptides for ODN or ribozyme delivery *in vivo*, a recent study using a model macromolecular drug, β-galactosidase, fused with the membrane-traversing Tat protein, resulted in the distribution of β-galactosidase in all tissues, including the brain, following intraperitoneal administration to mice[98]. This study suggested that carrier peptides could be useful for the improved delivery of macromolecules *in vivo* and that their use could also facilitate the delivery of ODNs and ribozymes across the blood–brain barrier (BBB). Their efficacy in the presence of serum proteins also suggests a potential for peptide–nucleic acid conjugates for *in vivo* applications.

CNS delivery

Traversing the BBB

Antisense technology has emerged as a useful tool for modulating gene expression in the CNS. Successful gene inhibition has been reported for a wide variety of targets, including trophic factors, ion channels and neurotransmitter receptors[99,100].

However, the large size and often polar nature of nucleic-acid drugs prevents these molecules from readily entering the CNS following systemic administration[6]. *In vivo* pharmacokinetic studies have demonstrated that <0.01% per cm^3 of a systemic injected dose of PS-ODN might reach the brain, where its residence time could be as short as 60 min[101].

Clinical techniques using hyper-osmolar agents, such as mannitol or arabinose, to permeabilize the BBB have proved unsuitable as they cause damage to healthy tissue. An alternative approach to target these compounds across the BBB is to 'parasitize' or 'piggy-back' onto the cell's own transport systems to facilitate delivery. The transferrin receptor, which is highly expressed in the brain capillary endothelium[102], has been widely studied as a delivery system. The OX26 monoclonal antibody is a murine antibody raised against the rat transferrin receptor, which undergoes receptor-mediated transport at similar rates to transferrin[103]. Boado and Pardridge[104] demonstrated that using a

PO-ODN with a single biotin residue at the 3′ terminus bound to a streptavidin–OX26 fusion protein they were able to deliver sufficient ODN *in vivo* to effect a complete inactivation of the target mRNA. However, when these experiments were repeated *in vivo* there was rapid degradation of the PO-ODN[105]. Using PO-ODN directly conjugated to OX26, it was also demonstrated that improved cellular uptake could be observed *in vitro* and that this uptake was facilitated by the transferrin receptor[106].

Local delivery

Unfortunately, the *in vitro* (cell culture) success of CNS delivery systems is largely non-transferable to *in vivo* models. A variety of factors could contribute to this, but the most probable reason is that ODNs and their constructs bind significantly to serum proteins. For example, when a PS-ODN was conjugated to a transferrin receptor-antibody delivery system and administered intravenously, Wu and coworkers[107] showed that high molecular mass complexes were formed by binding to plasma proteins. This substantial increase in the molecular weight of the delivery construct will prevent it from traversing the BBB.

A common approach to overcome this is the local introduction of antisense agents into the region of interest within the CNS, thereby circumventing the BBB[108,109]. Hence, many of the successful *in vivo* antisense studies in the CNS have involved either direct intracentroventricular or localized regio-specific injections[108,109]. However, repeated administration of some ODN chemistries have led to toxicity within the brain tissue[110]. As mentioned previously, the use of biodegradable polymer microspheres could potentially overcome these toxicity problems, and can also provide regio-specific and sustained delivery of ODNs[83].

Oral delivery

The oral route of drug delivery is relatively easy and painless and, as such, often has improved patient compliance over more invasive routes. There have been reports on the oral delivery of nucleic acids, the earliest being that by Vlassov and coworkers who demonstrated wide tissue distribution at pharmacologically active concentrations 20 min after administration[111]. Furthermore, Agrawal and colleagues[112] reported that oral bioavailability of up to 25% could be obtained when modified ODNs were administered to mice or rats. By contrast, oral administration of PS-ODN in a separate study[113] indicated a bioavailability of <1%. There could be several reasons for such differences, including the method of calculation used. However, it is clear that further studies are needed to resolve the controversy over these results.

More recently, effective oral delivery was reported when mice carrying a human colon cancer xenograft were treated with an ODN directed against the RIα subunit of protein kinase A[114]. The mice were dosed daily with the ODN at 10 mg kg^{-1}, either intraperitoneally or orally, and surprisingly similar antitumour effects were observed for up to 10 days post-administration via both routes. After 10 days, control mice treated with saline had an average tumour mass that was fourfold and twofold greater than the intraperitoneally and orally delivered ODN, respectively.

Because unmodified ODNs are rapidly degraded in the gastrointestinal tract, chemically modified nucleic acids are more likely to be effective by the oral route. However, a report by Moore and colleagues[115] suggested that ODNs conjugated with lipophilic groups such as cholesterol, because of their extensive and non-specific binding to cell surface proteins, could interfere with the normal functioning of some transport proteins. In particular, they showed a significant inhibitory effect of ODN conjugates on the functioning of the dipeptide transporter, an important mode of transepithelial transport for small peptides and some antibiotics. This area of possible drug interactions and compromising of normal transport functions also requires further investigation when considering the delivery of nucleic acids via the oral route.

Concluding remarks

This review has summarized some of the delivery strategies currently employed for improving the efficacy of antisense ODNs and ribozymes *in vitro* and *in vivo*. In reviewing the literature, it became apparent that nucleic acid delivery approaches in cell culture are likely to be different to those employed in whole animals. In cell culture, the major goals are to improve cellular uptake and trafficking, such that optimal amounts of nucleic acids reside in a bioavailable form within the cell to exert the desired pharmacological action. The most successful delivery approach in cell culture involves the use of cationic lipoplexes that contain facilitator or helper lipids, such as DOPE. When this system is optimized for dose, charge ratio, lipid type, cell type, ODN chemistry and length, these complexes can clearly enhance cellular uptake and promote ODN release from endosomal or lysosomal compartments. However, as most of the commercially available cationic lipid formulations remain unstable in serum, their successful application in whole animals has been limited.

To date, *in vivo* studies suggest that biological effects with naked ODNs are possible in the absence of a delivery enhancer[6]. This is often reconciled with the suggestion that uptake mechanisms, or at least intracellular trafficking, of ODNs *in vivo* might be different to those observed

in cell culture[7]. PS-ODNs clearly distribute to many tissues[6], but not to all cells *in vivo*. Recent *in vivo* biodistribution studies suggest that ODN uptake is heterogeneous and that different cells have a varying affinity for ODN uptake, a fact that is supported by several cell culture studies[116]. Direct administration of fluorescently-labelled PS-ODNs into an A431 tumour xenograft in nude mice resulted in only a subpopulation of cells displaying fluorescence, even within the area of ODN spreading[6]. This phenomenon was more apparent when similarly labelled ODNs were administered by direct injection into the neostriatum of the rat brain. Up to 24 h post-administration, the majority of naked ODN was localized within neuronal cells with little or no ODN present in even the immediately adjacent glial cells[83]. These findings suggest that delivery strategies *in vivo* could benefit from organ and cell-specific targeting. In addition, because of the relatively rapid elimination and excretion of some ODNs, strategies focusing on improving pharmacodynamics and pharmacokinetics would be useful; in this case, biodegradable polymeric delivery systems could play an important role. As we learn more about the pharmacokinetics and pharmacodynamics of these molecules it will be possible to refine our delivery strategies and make improvements in both their administration and delivery. Ultimately, effective delivery will lead to a more widespread use of both antisense ODNs and ribozymes as biological tools, drug-target validation agents and, hopefully, therapeutic agents.

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