Overcoming biological barriers to in vivo efficacy of antisense oligonucleotides

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Antisense oligonucleotides as a therapeutic platform have been slow to progress since the approval of the first antisense drug in 1998. Recently, there have been several examples of convincing antisense interventions in animal models and promising clinical trial data. This review considers the factors determining the success of antisense oligonucleotides as therapeutic agents. In order to produce target knockdown after systemic delivery, antisense oligonucleotides must avoid nuclease degradation, reticuloendothelial-system uptake and rapid renal excretion, and extravasate to the target cell type outside the vasculature. They then must enter the target cell, and escape the endosome–lysosome pathway so as to be free to interact with the target mRNA. We consider the significance of these limiting factors based on the literature and our own experience using systemic administration of antisense oligonucleotides.

It has been recognised for many years that antisense oligonucleotides – short single-stranded nucleic acid sequences that inhibit target gene expression via sequence-specific, Watson–Crick base-pair hybridisation to sense mRNA – can be employed to selectively reduce the expression of disease-related proteins in vivo, and consequently to improve disease phenotypes. A large number of biotechnology companies have emerged in that time with a view to translating the promise of 'antisense' in cell culture and animal models to successful pharmaceutical products. Target proteins as varied as the low-density lipoprotein (LDL)–cholesterol component apolipoprotein B100 (Refs 1, 2, 3), the skeletal muscle protein dystrophin (Refs 4, 5, 6), hepatitis C viral proteins (Ref. 7) and intracellular signalling molecules (often proto-oncogenes such as MYC; Refs 8, 9, 10) have all been shown to be 'knocked down' in animal models using antisense oligonucleotides and tested in clinical trials. Since the approval in 1998 of the first antisense drug, fomivirsen (Vitravene®; Refs 11, 12), which targets a cytomegalovirus mRNA, there has been a notable dearth of subsequent approvals for therapeutics of this type, due to a variety of factors preventing promising

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antisense candidates from reaching the clinic. Recently, however, there have been some positive clinical trial data from Isis Pharmaceuticals (Refs 1, 13), and several high-impact publications demonstrating excellent outcomes of antisense therapy in animal models of human disease (Refs 5, 14, 15, 16). Thus, it is timely to review the pharmaceutical science underlying the in vivo effects of antisense oligonucleotides. We focus here on the nature of the biological barriers to in vivo knockdown and strategies that have already overcome each barrier or may do so in the future, and then analyse the efficacy and potency of antisense therapeutics after intravenous administration.

Natural deoxyribonucleotides are not used as antisense oligonucleotides in vivo because of stability and delivery problems, and several chemical modifications have been introduced over the past decade to improve the efficiency of antisense therapeutics. The pharmaceutical chemistry of antisense oligonucleotides is reviewed elsewhere (Refs 17, 18, 19, 20, 21). The modifications considered in this article include: (1) first-generation phosphorothioate (PS) DNA antisense; (2) second-generation 2′-*O*-alkyl (particularly 2′-*O*-methoxyethyl; MOE) 'gapmer' chimaeric DNA–RNA antisense (Ref. 21); (3) phosphorodiamidate morpholino (PMO) antisense (Ref. 22); (4) peptide nucleic acid (PNA) (Ref. 23); and (5) locked nucleic acid (LNA) antisense (Refs 18, 24). These modifications and others have achieved the goal of producing nuclease-resistant antisense agents that retain high efficacy in vitro. 2′-*O*-modification and LNA chemistries do not support RNase H activity, and oligonucleotides containing these chemistries therefore require regions free of 2′-modification to recruit this high-efficiency silencing mechanism. Monia and colleagues demonstrated that a region containing five or more 2′-deoxy nucleotides was required for RNase H activity (Ref. 25). A major innovation allowing the benefits of MOE, 2′-*O*-methyl (OMe) and LNA chemistries to be realised without the loss of efficacy has thus been the use of gapmer strategies (Refs 25, 26, 27, 28) in which central regions of the oligonucleotide consisting of nucleotides whose chemistry supports RNAse H activity – that is, unmodified phosphodiester (PO) internucleoside linkages (PO or PS) – are flanked by 2′-modified regions (eg. MOE–PS–MOE). PMO oligonucleotides differ from the other modifications described above in that these agents are designed to work via steric inhibition of translation rather than degradation of mRNA transcripts.

This review focuses mainly on the barriers to efficacy of second- and third-generation antisense oligonucleotides in vivo, with a view to informing the use of antisense as both a research tool in animal models and a therapeutic platform in humans. Much of the research described herein is from animal studies that have revealed insights into antisense mechanisms; where informative human data are available, these have also been included.

General pharmacokinetic properties of antisense oligonucleotides

Antisense oligonucleotides exhibit predictable pharmacokinetic properties given their size and charge. They can exist as free molecules within the plasma or exhibit strong plasma protein binding depending on sequence and modification. Antisense oligonucleotides almost universally exhibit biphasic plasma concentration–time profiles, usually with a rapid initial decrease in plasma concentration within the first hour after intravenous injection in mice (Ref. 29), rats (Ref. 30) and humans (Ref. 29) ($t_{1/2}\alpha$ of around 3–30 min), even for nuclease-stable antisense (Refs 31, 32), and a slower second phase of clearance over the next 24 h ($t_{1/2}\beta$) (Refs 30, 33). In humans, a similar pharmacokinetic profile is observed for PS oligonucleotides (Refs 33, 34, 35). A cross-species comparison for ISIS 301012 found only small differences between the pharmacokinetic properties of the drug in mice, rats, monkeys and humans (Ref. 29). For nuclease-stable oligonucleotides, the biphasic nature of the fall in plasma concentration represents a combination of renal excretion, tissue distribution and tissue clearance, considered in the following sections.

Tissue distribution

The kidney and liver are the predominant destinations for antisense oligonucleotides. More than 80% of the antisense oligonucleotide detected in tissues after intravenous administration is found in these two organs, and these observations are valid for both charged (Ref. 31) and uncharged (Ref. 30) antisense oligonucleotides, and also after oral

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(Refs 32, 36) or intradermal (Ref. 36) delivery. When the spleen is included in the analysis, the proportion of dose within these tissues rises above 90% in many cases.

Table 1 shows representative examples of tissue distribution for oligonucleotides of varying chemistries. The major determinants of tissue distribution remain unclear to some extent, but blood flow, specific cellular uptake (e.g. reticuloendothelial function) and preference for organs with fenestrated vascular endothelia all appear to be relevant factors, as considered further in the next section. The liver and kidney receive large proportions of cardiac output, and these organs accumulate significant proportions of an administered oligonucleotide dose, with the kidney predominant for all chemical modifications other than PS (Table 1). However, oligonucleotide distribution is not always directly proportional to the blood flow to that organ. Collectively, skeletal muscle receives >20% of cardiac output in mice (Ref. 37) and rats (Ref. 38), yet retains <5% of oligonucleotide across a variety of modification types and experimental protocols (Table 1), and the heart and gastrointestinal tract also show discordance between the proportion of cardiac output received and the proportion of oligonucleotide they contain (Table 1). Thus, there is preferential distribution of oligonucleotide to liver, kidney and spleen (and bone marrow) at the expense of other tissues.

Table 1. Tissue distribution of the major classes of modified antisense oligonucleotides after systemic administration[a]

Modification (Ref.)	Dose (mg/kg)	Time of tissue collection (min)	Kidney	Liver	Spleen	Heart	Intestine	Skeletal muscle
Mouse								
PS (Ref. 39)	0.8	90	20[c]	180	20	<5	NR	NR
MOE gapmer (Ref. 40)	5	1440	90[c]	20	20	<5	<5	NR
LNA (Ref. 72)	6	1440	175[d]	15	<10	<5	5-10	<1
Rat								
PS (Ref. 44)	0.04	120	13[c]	15	5	<2	<2	<1
LNA mixmer (Ref. 58)	0.01	120	15[c]	40	30	<2	<2	<2
PMO (Ref. 48)	15[b]	1440	10[e]	20	10	5	NR	NR
PNA (Ref. 30)	5	120	180[d]	10	5	5	NR	NR
Monkey								
MOE gapmer (Ref. 31)	10	1440	300[d]	25	<10	NR	<5	NR

[a]From each article, we extracted the following information: the dose used in animals ([b]daily injections for 5 days), the time of tissue collection and the concentration of drug in each tissue. To allow comparison, we normalised the tissue concentration data to those of the kidney, which for the majority of studies contained the greatest concentration of oligonucleotide. Values are approximates that we calculated from figures within the cited articles, expressed as % dose[c], concentration[d] (μg/g) or micromolar concentration[e].
Abbreviations: LNA, locked nucleic acid; MOE, 2′-*O*-methoxyethyl; NR, not reported; PMO, phosphorodiamidate morpholino; PS, phosphorothioate.

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Tissue clearance of oligonucleotide

The clearance of oligonucleotide from the liver, kidneys and spleen is slow, and due in large part to nuclease degradation. The tissue half-life of most nuclease-resistant oligonucleotides is extremely long, with reported values generally expressed in days rather than hours (Refs 39, 40). The rate of clearance, and resultant duration of action of the oligonucleotide, is dependent on the modification, with MOE oligonucleotides demonstrating tissue half-lifes greater than 10 days across a number of tissues and across species (Ref. 40).

Barriers to efficacy of antisense therapeutics

Overview

In order to gain access to complementary mRNA species within tissues after systemic administration, antisense oligonucleotides must avoid nuclease degradation, reticuloendothelial system uptake and renal excretion, traverse the microvascular endothelium, cross target cell membranes and escape the endosome–lysosome system (Fig. 1). The combination of these barriers has contributed to the requirement for larger doses to be used in animal experiments and clinical trials, and thus greater class-related and drug-specific toxicity. The failure of antisense oligonucleotides in clinical trial has resulted from deficiencies of both efficacy and safety (Ref. 41). In many cases it has been difficult to measure target knockdown as an indicator of efficacy. For those cases where clinical efficacy has been lacking, it is often difficult to assign this to a failure of stability, delivery to the target cell type, uptake, endosomal escape, mRNA cleavage, or choice of the therapeutic target. For example, the development of GEM91 by Hybridon was halted when dose-limiting thrombocytopaenia occurred in Phase II trial, but in any case the efficacy seen in Phase I trial (as indicated by levels of viraemia) was not replicated in the Phase II trial according to a press release (Ref. 42) – greater understanding of the fate and efficacy during the development of GEM91 may have prevented the failure or indicated that development should be halted at an earlier point. Where target knockdown can conveniently be correlated with disease outcomes, such as for ISIS301012 (Refs 1, 13), the close evaluation of efficacy during each stage of development allows for more informed drug development.

Barrier 1: nuclease degradation

Studies using radiolabelled phosphodiester antisense oligonucleotides have shown that these agents are likely to have little utility in vivo because of rapid nuclease degradation (Refs 43, 44, 45), with $<15\%$ of intact drug detectable in rat plasma 20 min after dosing (Ref. 44). The major modified antisense chemistries have overcome this problem to a large extent. PS oligonucleotides (Ref. 43), MOE–PS–MOE gapmers, LNA oligonucleotides (Ref. 46) and LNA–DNA–LNA gapmers (Ref. 26) are stable in serum for extended periods, whereas OMe–PS–OMe gapmers show moderate serum stability in vivo (Ref. 47). A study using a gel-shift assay to detect degradation of PNA in rat tissue and urine samples after intravenous administration found no detectable degradation products (Ref. 30). PMO oligomers show good serum stability in rats after intravenous injection, as does the PMO portion but not the cell-penetrating peptide portion of PMO–cell-penetrating peptide conjugates (Ref. 48). The extent of modification is an important factor here, with fully modified PS being more stable than partially modified PO/PS sequences (Ref. 47).

Barrier 2: clearance from the blood by the kidney and reticuloendothelial system

Clearance via the kidney

The degree of renal excretion of antisense oligonucleotides varies widely depending on the chemical modification, dose and species. Unmodified oligonucleotides are rapidly metabolised and degradation products renally excreted (Ref. 26). PS oligonucleotides often display far lower rates of renal clearance; some PS sequences are able to avoid glomerular filtration by binding to plasma proteins such as albumin (Ref. 49). The degree of renal excretion has been reduced to a low level using PS antisense oligonucleotides selected for high plasma-protein-binding affinity; two antisense molecules that exhibited $>90\%$ plasma protein binding showed slow plasma clearance in monkeys, while an antisense with $<80\%$ plasma protein binding exhibited rapid plasma clearance (Ref. 31). This study also demonstrated

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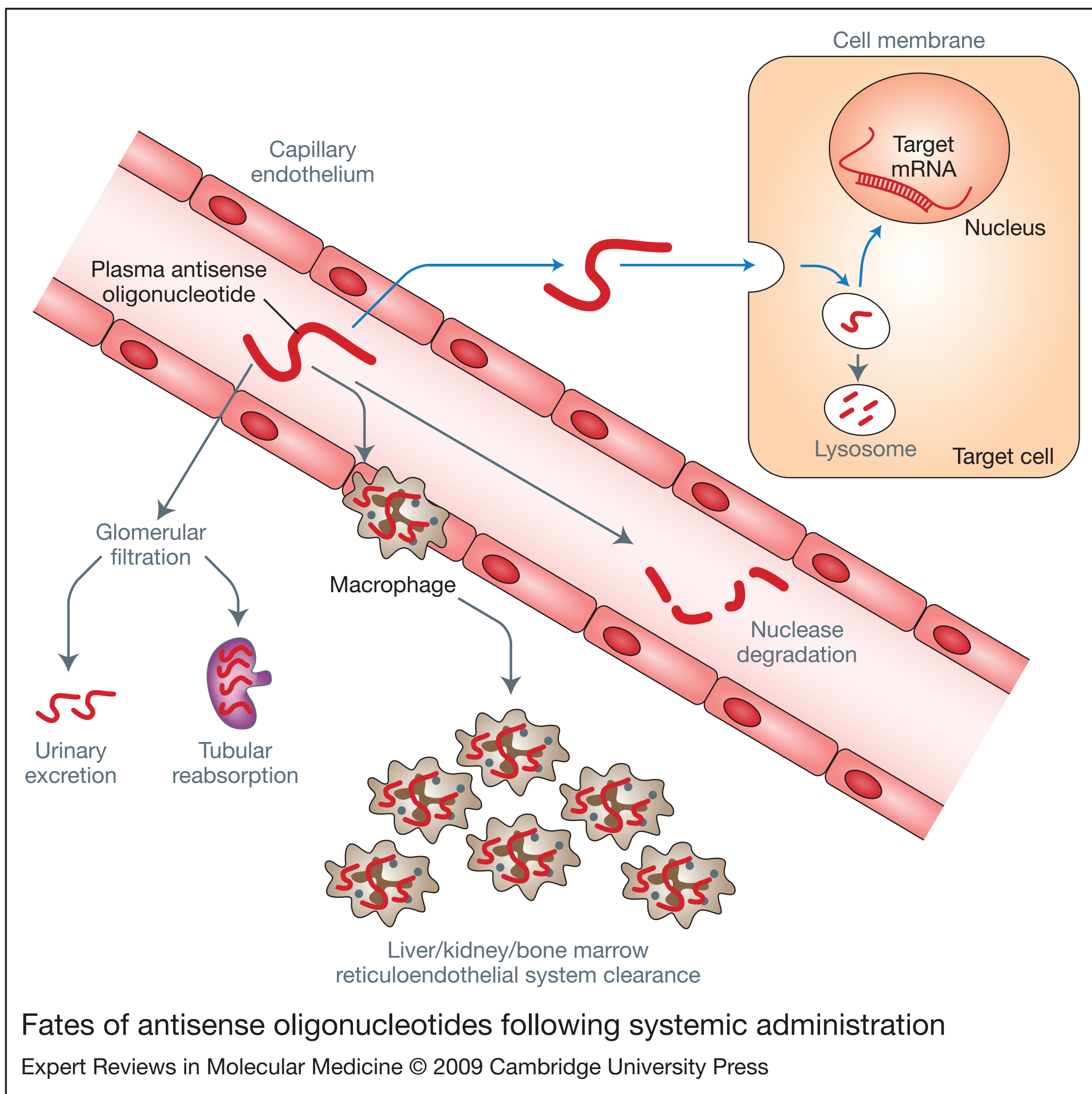

Figure 1. Fates of antisense oligonucleotides following systemic administration. (a) Successful gene silencing after systemic administration requires the movement of the antisense oligonucleotide from the blood into the interstitium, then into the target cell (nucleus) to reach the target mRNA (blue arrows). (b) However, there are several barriers and mechanisms to prevent successful knockdown (grey arrows and grey labels). Antisense oligonucleotides may be degraded in the blood, or removed by renal excretion or uptake by tissue macrophages. Antisense oligonucleotides may not reach their target cells because they are unable to escape across the vascular wall. Once in the interstitium, antisense oligonucleotides may be prevented from reaching their intracellular target (mRNA) by an inability to cross the cell membrane or escape the endosome–lysosome pathway.

that, in mice, the proportion of PS oligonucleotide excreted in the urine increased at doses >5 mg/kg, suggesting saturation of albumin binding. Furthermore, MOE gapmers with increasing proportion of phosphodiester internucleoside linkages show increasingly greater renal excretion than those containing fully phosphorothioated central regions (Ref. 50). It is pertinent to emphasise that not all PS oligonucleotides share the property of low renal clearance: an early study

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in humans found that a low dose (0.1 mg/kg) resulted in ~50% urinary excretion within 24 h in human immunodeficiency virus (HIV) patients (Ref. 33), possibly as a result of a sequence that exhibits low affinity for albumin. Nonetheless, PS oligonucleotides can be developed to exhibit low urinary excretion in humans: <10% of the administered dose of ISIS 104838 (Ref. 34) and ISIS 2302 was found in urine 24 h after intravenous dosing, and the low level of urinary excretion is retained in the MOE gapmer ISIS 301012 in humans (Ref. 29). Methodological differences may account for some of the variability in reported plasma levels and urinary excretion; for example isotope choice and placement can affect the quantitation of urinary excretion rates for radiolabelled drug (Ref. 39).

Non-PS oligonucleotides generally exhibit greater urinary excretion than PS oligonucleotides. Urinary excretion of OMe–PS–OMe gapmer oligonucleotides has been shown to be extensive (30–50%) over 72 h (Ref. 47), although this study employed a high dose, which may have increased the level of urinary excretion as discussed above. PNA oligomers appear to undergo rapid and extensive urinary clearance: in rats, >90% of a 5 mg/kg intravenous dose was recovered as parent drug in the urine (Ref. 30), although this characteristic may not hold for PNAs containing positively charged residues (Ref. 51). A comparison of LNA and PS oligonucleotide urinary excretion (Ref. 46) revealed far greater renal excretion of the LNA than the PS 30 min after intravenous injection in nude mice. Although the albumin-binding affinities for these modifications are often untested, it is possible to speculate that a lower affinity for albumin was responsible for the rapid urinary excretion of LNA oligonucleotides. For chemistries with low albumin-binding affinity, strategies that reduce renal excretion, including particle-based approaches, will be expected to increase the efficacy of the drug.

Uptake by cells within the kidney

The amount of drug retained within the kidney is of interest both from the perspective of targeting kidney disease and in relation to renal oligonucleotide toxicity. The mechanisms of uptake of antisense into proximal tubule epithelial cells (the major cellular location for oligonucleotides within the kidney) (Refs 52, 53) are worth consideration, particularly given the differing importance of renal excretion for PS and non-PS oligomers described above. Proximal tubule epithelial function is important in renal oligonucleotide accumulation, as the use of cisplatin to disrupt this activity has been shown to decrease oligonucleotide concentration in the renal cortex (Ref. 54). For LNA, PNA and PMO oligonucleotides, the exposure of the proximal tubule epithelium to high concentrations of drug in the filtrate would be expected to result in significant uptake in this actively sampling cell type.

An interesting conceptual problem arises for PS oligonucleotides: given the extremely low levels in the urine for first- and second-generation oligonucleotides with high albumin-binding affinity, how do the proximal tubule cells gain access to the drug? Clearly, access is possible, in that the renal cortex usually contains >20% of an administered dose (Table 1), localised to granular structures within proximal tubule epithelial cells (Ref. 55). The answer appears to be that both basolateral uptake from the blood and tubular reabsorption from the filtrate contribute to PS uptake into proximal tubule epithelial cells (Refs 53, 56), with the latter predominant (Ref. 57). The ratio of Bowman's capsule to plasma concentration of ^{35}S-labelled PS oligonucleotide has been found to be low in rats (Ref. 53), indicating that filtration of these agents was limited to some extent. However, tubular reabsorption of PS oligonucleotide did 'account for the large amount of oligonucleotide found in the kidney after intravenous infusion' (Ref. 53). In isolated, perfused rat kidneys under nonfiltering conditions, PS oligonucleotide absorption from capillaries has been observed; furthermore, this absorption was reduced in the presence of polyanions (Ref. 49). The fraction absorbed from the capillaries constituted only a small fraction, however, of the total renal uptake in filtering conditions for PS oligonucleotides. One conclusion to be drawn from these data is that a small but significant amount of PS oligonucleotide (the unbound fraction in the albumin–oligonucleotide equilibrium) is, in fact, filtered and that efficient tubular reabsorption of this

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filtered drug accounts for most of the MOE–PS–MOE gapmer in the renal cortex after intravenous administration.

Uptake by the reticuloendothelial system

The reticuloendothelial system, consisting of mononuclear leukocytes that circulate in the blood and enter tissues to become scavenging macrophages, appears to play an important role in the removal of antisense oligonucleotides from the blood. After systemic administration, high concentrations of PS, PO, LNA hybrid oligonucleotides and OMe oligonucleotide are found in bone marrow (Refs 44, 58, 59) (in particular in large nucleated cells within the red bone marrow; Ref. 60), within the spleen (Refs 40, 58) (in particular in the red pulp of the spleen; Ref. 60) and in resident macrophages (Kupffer cells) in the liver (Ref. 61). These findings suggest that macrophage-mediated uptake of oligonucleotide plays an important role in the tissue distribution of these agents. Within the liver, Kupffer and endothelial cells show high levels of PS oligonucleotide after intravenous injection: a study in rat liver showed that 80% of the total organ cellular PS oligonucleotide dose was located within these cells, whereas hepatocytes exhibited relatively poor uptake and, despite the larger number of hepatocytes, only 20% of the liver-associated oligonucleotide was found in this cell type (Ref. 60). Higher doses of oligonucleotide may be required to cause antisense inhibition in hepatocytes compared with Kupffer cells (Ref. 61). Uptake of PS oligonucleotides by cells of the reticuloendothelial system has been suggested to be dependent on 'scavenger receptor' binding. Nonspecific anionic inhibitors of scavenger receptors such as dextran sulphate impair hepatic and splenic uptake of PS oligonucleotide in a dose-dependent manner (Ref. 57). However, comparison of mice deficient in the predominant candidate scavenger receptor (SR-A) with wild-type mice shows either no difference or modestly reduced cellular uptake (Kupffer cells) of PS oligonucleotides (Ref. 62). The specific proteins responsible for cellular uptake in vivo across different cell types remain elusive.

A variety of delivery strategies have since been successful in avoiding or reducing uptake of antisense oligonucleotides by the reticuloendothelial system. Cholesterol conjugation of PS oligonucleotide results in impressive targeting to the liver such that it contains about 80% of the administered dose, and a redistribution to endothelial cells and hepatocytes rather than Kupffer cells is observed (Ref. 63). However, uptake of liposomally encapsulated oligonucleotide by the reticuloendothelial system is enhanced compared with free oligonucleotide (Ref. 64). This altered disposition was used to advantage (Ref. 65) to deliver TNF-α antisense to macrophages in vivo. These studies demonstrate that shifting between a predominantly reticuloendothelial system and a predominantly hepatic parenchymal distribution is possible. What is still lacking in the literature is a reliable delivery system that results in avoidance of both reticuloendothelial system and hepatic uptake such that the concentration in other organs increases.

Barrier 3: extravasation in organs other than liver, kidney and spleen

One aspect of antisense oligonucleotide pharmacokinetic behaviour that is often ignored is the relative ability of these molecules to escape the vasculature. The microvascular endothelium, in organs other than the kidney and liver, consists of a cell monolayer with close intercellular connections, which excludes relatively large hydrophilic species such as antisense oligonucleotides as a result of their inability to partition across the lipid regions of the cellular membrane or to penetrate the tight junctions between the endothelial cells.

The importance of extravasation as a barrier to antisense efficacy has not been directly evaluated. The low concentration within lymph nodes in mice after intravenous injection of up to 100 mg/kg PS oligonucleotide observed previously (Ref. 39) suggests that extravasation might be a limiting factor for these agents. Other indirect evidence may be inferred from the lack of partitioning of antisense oligonucleotides into organs other than the liver, kidney and spleen, which have either fenestrated or sinusoidal endothelia. However, this argument may be countered by the fact that specific mechanisms described earlier (macrophage and renal proximal tubule epithelial uptake) may be largely responsible

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for the high concentration of drug retained within these tissues.

For organs such as the heart, antisense strategies will require enormous efficacy from their therapeutic agents, as generally <5% of the systemically delivered dose reaches and/or is retained within these organs (Table 1). For tissues where the drug access is very poor, in particular the central nervous system, it appears very unlikely that systemically delivered antisense oligonucleotides will have a major role to play in future therapeutic strategies without delivery systems that enhance access to target cells. An exception might be made for PNAs, which have been demonstrated to reach and produce measurable antisense effects in the central nervous system (Ref. 66) after intraperitoneal administration – a finding attributed to an ability to partition across the blood–brain barrier due to lack of charge on the molecule. Tumours represent an atypical tissue type in many respects, including microvascular barrier function. Tumours are supplied by altered microvasculature, which allows for increased access to and retention of macromolecules (Ref. 67). In humans, PMO oligomers were found to reach significant concentrations within breast and prostate tumours after a single 90 mg dose, with tumour drug concentrations similar to in vitro knockdown IC_{50} values (Ref. 8).

A theme that emerges when considering barriers to antisense efficacy in vivo is that successes in the antisense field appear to involve (1) choice of targets that play to the strengths of the pharmacokinetics and tissue distribution of antisense oligonucleotides, or (2) strategically using chemical modification or delivery strategies that alter pharmacokinetics or tissue distribution towards a favoured target.

Barrier 4: cellular uptake

The ability of antisense oligonucleotides to produce target knockdown is of course also dependent on the level of uptake into the target cell. An observation from animal studies is that, unlike cultured cells, several cell types in vivo take up oligonucleotide via a process that results in high nucleus to cytoplasm concentration ratios (Refs 60, 68). Keratinocytes avidly take up oligonucleotide in a manner that results in nuclear localisation after intradermal injection in vivo (Ref. 68) but not in vitro (Ref. 69), and the same distribution pattern has been observed in freshly isolated gastric smooth muscle cells in the absence of transfection reagent (Ref. 70). It is clear that if sufficient antisense oligonucleotide can gain access to many cell types in vivo, there is a good chance for cellular uptake and successful target knockdown in that tissue. Furthermore, an in vitro study modelling the barriers to antisense efficacy found that intracellular trafficking and the affinity for target mRNA may be of greater significance than total cellular uptake (Ref. 71). Although promising, these results do not demonstrate that efficacy in a particular organ is assured when significant tissue drug concentrations are observed. For example, splice correction has been demonstrated using LNA oligonucleotides in liver and gastrointestinal tract but not in kidney – the organ where by far the greatest accumulation of drug occurred (Ref. 72). Delivery strategies that allow for cellular entry per se are not necessarily effective, but those that promote cellular entry in a manner that avoids subsequent endosome–lysosome trapping are likely to be very productive, as described in the next section. A greater understanding of cellular uptake mechanisms for free antisense oligonucleotide is important for continued development of antisense strategies.

Cell-penetrating peptides have been used to promote the cellular uptake of antisense oligonucleotides. Although these agents have shown great promise, and some in vivo efficacy has been demonstrated using conjugation with Tat from HIV (Refs 73, 74), it is not apparent at this stage that cell-penetrating peptides provide a robust system for cellular uptake of antisense oligonucleotides. Methods that produce cell-type-specific uptake of antisense oligonucleotides have long been sought, with little success. In this regard, the field of small interfering RNA (siRNA; RNA duplexes that cause silencing via RNA interference) has perhaps developed at a greater rate than that of antisense oligonucleotides (Ref. 75).

Barrier 5: endosomal escape and particulate strategies to enhance delivery of antisense oligonucleotides

The endosome–lysosome pathway is clearly a major limiting factor to access of the

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oligonucleotide to mRNA. Cationic polymers and cationic liposomes have been used extensively in cell culture to allow antisense oligonucleotides to escape endosomes and gain access to target mRNA. However, the systemic administration of cationic particles and complexes, such as lipoplexes, leads to rapid uptake by macrophages (Ref. 65). Approaches using polyethyleneimine alone have been successful in models of inflammatory disease (Ref. 76). Moderate success has been reported with shielding of the polycation from the reticuloendothelial system facilitated by a surface coating of polyethyleneglycol (Ref. 77).

Future directions: overcoming barriers to antisense oligonucleotide

Attempts to overcome the biopharmaceutical barriers to systemic administration of antisense therapeutics have therefore developed towards rational design of multifunctional antisense oligonucleotide delivery systems – either as colloidal particles held together by electrostatic attraction or using medicinal chemistry advances to produce novel modified oligonucleotides. The goals in each case are to provide one or more of the following: improved pharmacokinetic profiles, and selective targeting to particular tissues through modification of the particle surface with ligands that allow targeting, cellular uptake and endosomal release. A schematic of such a colloidal construct is shown in Figure 2.

Polymer-based nanoparticles are a colloidal system that has received recent attention for delivery of antisense; of particular interest are particles prepared using biodegradable polymers such as poly(D,L-lactide-co-glycolide) (Ref. 78), which can be used to release therapeutic agent slowly as the polyester is degraded by hydrolysis. Chitosan, a cationic biopolymer that can be prepared in nanoparticle form, has also been shown to be useful for encapsulation and cellular uptake of antisense oligonucleotides in vitro (Ref. 79). Polycationic dendrimers are colloid-sized polymeric constructs that in some instances have been shown to be biodegradable in vivo (Ref. 80) and are able to induce the so-called 'proton sponge' endo-osmotic disruption effect. Briefly, by acting as weak bases once inside the acidic endosome environment, these agents act to increase osmotic pressure across the endosomal membrane and induce swelling and rupture of the endosome, leading to release of organelle contents. They have been employed as electrostatically associated carriers for oligonucleotides, and in vitro studies using dendrimers have shown antisense delivery and reduced luciferase expression in luciferase-expressing cell lines (Ref. 81) Further functionalised dendrimers with targeting and moieties that extend the circulatory half-life of the dendrimers (Ref. 82) indicate strong potential for dendrimers, incorporating all of the functionality of the model system in Figure 2, to overcome the barriers currently limiting extravasation and delivery of antisense in vivo. Derivitisation of polycations such as chitosan, cationic dendrimers, polyethylineimine, and cationic poly(amino acid)s is likely to be required if the intention is to use polyelectrolyte complexes as the core of a delivery system for intravenous use. Although complexes with excess cationic charge can be used to deliver antisense therapeutics in vitro, the result of injecting such complexes intravenously is substantial uptake by the reticuloendothelial system, as well as the threat of particle aggregation following interaction with blood components. When the latter occurs, the result is usually deposition of the particles in the capillary bed of the lung. These phenomena have been well documented in the field of nonviral gene therapy – that is, using cationic polymer (Refs 83, 84, 85) or lipid complexes (Refs 86, 87, 88, 89) to deliver DNA expression plasmids. Although siRNA delivery is beyond the scope of this review, recent studies in this area (Ref. 90) suggest that small nucleic acid molecules can be delivered in neutral particles (Refs 91, 92) or as direct chemical conjugates with targeted neutral polymers (Refs 93, 94), and it is likely that similar approaches can be effective using antisense oligonucleotides. An important aspect of the use of colloidal systems to deliver antisense oligonucleotides is that the toxicity of the components of the system may be a limiting factor, as has been the case in the past with cationic liposomes.

Table 2 summarises current successes in overcoming the barriers to antisense oligonucleotide delivery to the cytoplasm of target cells, along with suggested directions for future work. At the time of writing this article, Juliano and colleagues (Ref. 75) published a

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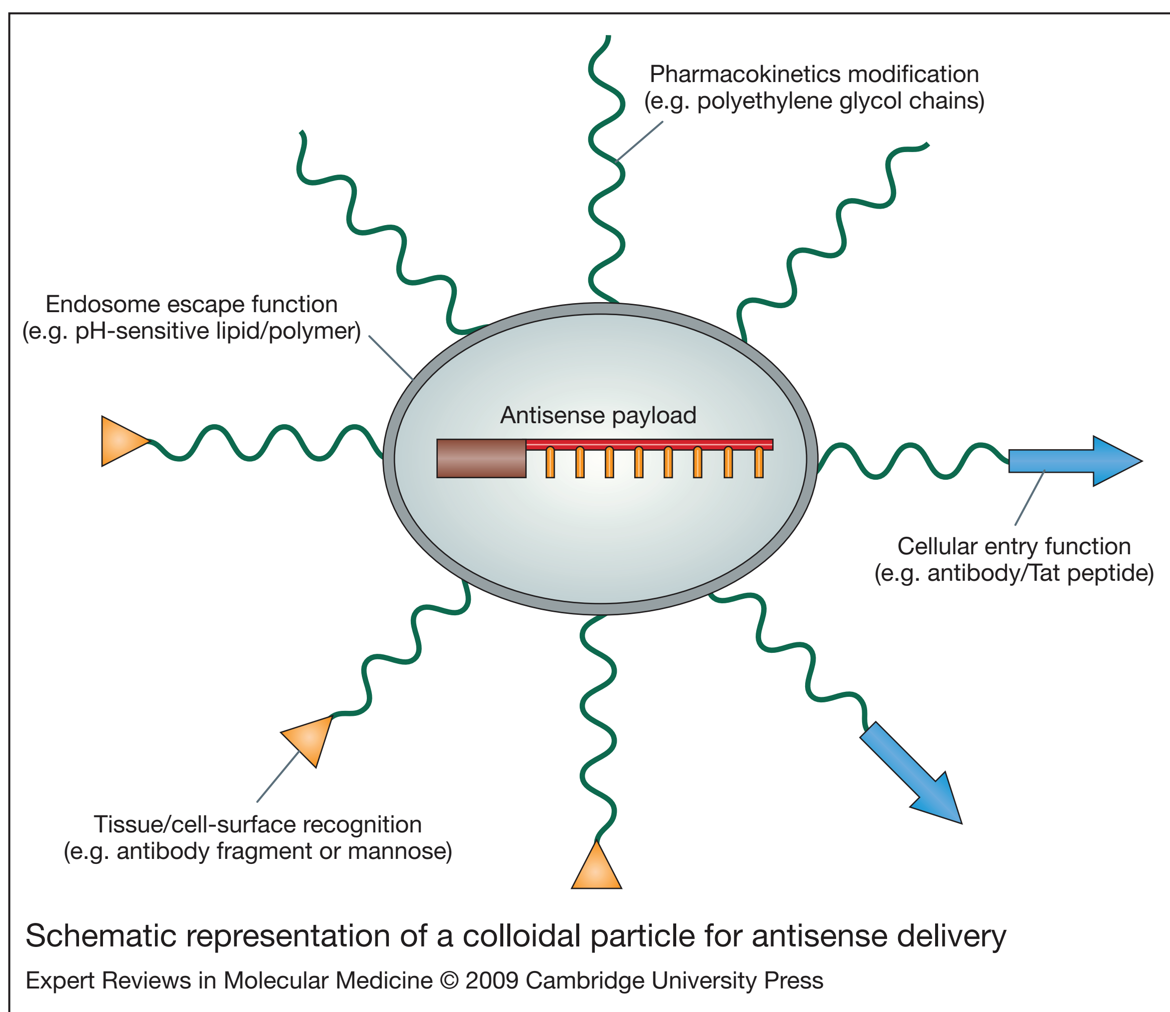

Figure 2. Schematic representation of a colloidal particle for antisense delivery. This diagram shows an ideal construct that carries antisense oligonucleotides (protected from degradation), is able to circulate without reticuloendothelial system uptake, and has targeting, cell-penetrating and endosomal escape functionalities.

similar list of barriers to oligonucleotide delivery: there is agreement as to the problems faced – the remaining questions concern the respective solutions.

Successful antisense knockdown after systemic administration

Although the list of barriers described earlier appears imposing, antisense oligonucleotides have been shown to reduce target expression and alter disease phenotypes in many well-controlled in vivo experiments. A great number of significant publications have emerged over the past 20 years describing antisense-mediated efficacy in vivo after systemic administration using simple injection of oligonucleotide-containing solutions. The most convincing of these combine most or all of the following properties: characterisation of the knockdown or exon skipping in vitro using robust controls [see review by Stein and Krieg (Ref. 95) for interpretation of antisense data], low doses of antisense oligonucleotide, and clear evidence of protein knockdown in vivo correlating with the observed phenotype change.

Table 3 summarises the doses used (mg/kg) and efficacy observed for selected examples of antisense efficacy in vivo across a range of target organs. It is clear that efficacy can be seen at low mg/kg doses, and that the maximum efficacy

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Table 2. Barriers faced by antisense oligonucleotides when given systemically, and approaches for overcoming them[a]

Barrier	Significance	Published strategies to overcome barrier	Future directions
Nuclease degradation	High	Chemical modification (Refs 26, 30, 43, 46)	None required
Renal excretion	High	Phosphorothioate modification, selection of sequences that result in high albumin-binding affinity (Refs 31, 34, 49)	Particulate strategies to avoid glomerular filtration for nonphosphorothioate
Reticuloendothelial system	High	Cholesterol conjugation (Ref. 126)	Stealth particles coated with inert polymers such as polyethyleneglycol
Extravasation	Unclear	None	Functionality to enable transcytosis across vascular endothelium
Cellular uptake	Cell-type dependent	Cell-penetrating peptides (Refs 73, 74)	Targeting functionality (antibody) to produce cell-type-specific uptake
Endosomal escape	Moderate–high	Proton-sponge endosomal escape moieties (Ref. 81)	Nontoxic endosomal escape

[a]The table is a chronological listing of the barriers faced by antisense oligonucleotides when given systemically, and approaches that may be employed to overcome these barriers. The relative importance of each barrier is rated and existing strategies to overcome the barrier identified where possible.

Table 3. Representative examples of the determination of maximal efficacy of in vivo knockdown[a]

Organ	Ref.	Dose (mg/kg)	Target	% Knockdown	Chemistry	Species
Liver	101	5–50	ApoB	~90[b]	MOE gapmer	Mouse
	72	25	EGFP	85	LNA	Mouse
Tumour	127	75	Survivin	60–80	PS	Mouse
Kidney	102	10	CTGF	83	MOE gapmer	Mouse
Skeletal muscle	5	~100[c]	Dmd	≤30	PMO	Mouse
Heart	98	1.5	EGFR	50	PS + liposome	Rat
	99	1	B_1 AR	30–50	PS + liposome	Rat

[a]Data were collected from original research articles, across a range of target organs showing doses used and the percentage decrease in expression of target mRNA or protein (% knockdown).
[b]The first reported measurement in this study was 25 days after initiation of therapy.
[c]Approximate; authors used 2 mg per mouse.
Abbreviations: ApoB, apolipoprotein B; B_1 AR, β_1 adrenoceptor; CTGF, connective tissue growth factor; Dmd, dystrophin; EGFR, epidermal growth factor receptor; EGFP, enhanced green fluorescent protein; LNA, locked nucleic acid; MOE, 2'-*O*-methoxyethyl; PMO, phosphorodiamidate morpholino; PS, phosphorothioate.

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reported is greatest in the liver and kidney. The heart was chosen to represent tissues identified earlier in the review that show modest levels of tissue drug concentration. Although many groups including ourselves (Refs 96, 97) have shown antisense efficacy in the myocardium, the ceiling on the maximum possible level of target suppression appears to be around 50%, even when delivery strategies such as liposome encapsulation are used (Refs 98, 99). Similarly, correction of aberrant splicing in skeletal muscle has required large doses of antisense to produce relatively low levels of correction (Refs 5, 100), although this may be enough to cause phenotypic improvement, and in these impressive studies many muscle fibres show evidence of correction. Correction of aberrant splicing in the liver by the same investigators was more successful, with 85% correction (Ref. 72) at lower doses than used in skeletal muscle. Maximum levels of knockdown in cardiac and skeletal muscle therefore appear to be lower than that for the liver, in which up to 90% silencing has been shown in vivo (e.g. Ref. 101), and the kidney, in which 83% knockdown in the proximal tubules has been shown (Ref. 102). Thus, the literature appears to suggest that significant enhancement of tissue and cellular uptake using delivery systems is required for high-efficacy silencing in tissues such as the heart and skeletal muscle.

While a comprehensive coverage of successful employment of antisense oligonucleotides as research tools is beyond the scope of this review, it is pertinent to consider some key examples of convincing in vivo data in which the experimental controls were rigorous and the efficacy noteworthy. We direct the reader to examples of local or systemic injections of a range of antisense oligonucleotides (PS, OMe and MOE gapmers, PMO oligonucleotides and LNA antisense) which were shown to produce specific target mRNA knockdown (or exon skipping) (see Refs 51, 96, 100, 101, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112) in a range of tissues (liver, gastrointestinal tract, brain, skeletal muscle, vasculature) (see Refs 51, 96, 101, 103, 104, 107, 110) and against a range of targets (enzymes, intracellular signaling molecules) (see Refs 9, 104, 107, 108, 112) in order to reverse disease phenotype.

Outstanding examples of impressive antisense effects in vivo using simple injection not surprisingly relate to organs with naturally leaky vascular beds – in particular the liver and in tumours (Ref. 113). Apolipoprotein B knockdown has been reported in carefully controlled studies in animals (Ref. 101) and in dyslipidaemic humans (Ref. 114) using moderately low doses of ISIS antisense oligonucleotides, and the future of this strategy appears to be promising. Tumour regression and inhibition of metastasis by targeting a kinase suppressor of Ras (Ref. 113) has been elegantly demonstrated – a case in which specific intracellular signalling molecules are critical for tumour development and where gene-silencing approaches may be more useful than traditional small-molecule strategies. Conferring of positive charge on PNAs, using lysine (K) residues at the C-terminus, greatly improves the drug distribution to a range of tissues after intraperitoneal injection (Ref. 51). In this study, 'PNA-4K' antisense oligonucleotides demonstrated efficacies (correction of aberrant splicing in this case) equal to or greater than MOE antisense oligonucleotides in a range of organs, whereas uncharged PNA or PMO antisense oligonucleotides were relatively ineffective.

Direct measurement of in vitro target mRNA knockdown (or exon skipping) and/or subsequent target protein knockdown is often not possible in humans due to the inability to excise and collect target tissues for measurement of mRNA levels. As a result, reversal of disease phenotype is often the only measure of antisense drug efficacy and this is, at best, only an indirect measure of target mRNA knockdown. This is demonstrated by the first antisense drug to reach the market, fomivirsen, for which clear evidence of clinical efficacy (Refs 115, 116) but no direct evidence of antisense-mediated target knockdown in humans can be found in the literature. Furthermore, only a limited number of antisense-oligonucleotide-based therapeutics have entered clinical trials, with the vast majority being of PS chemistry and with most studies reported being Phase I trials in which safety, rather than efficacy, was the primary endpoint (Refs 34, 117, 118, 119, 120, 121, 122, 123). Later-phase studies with a PS oligonucleotide targeting BCL2 mRNA returned promising results as a cancer therapy adjuvant in humans (Refs 124, 125); however, these studies did not directly measure target mRNA

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or protein knockdown. One excellent example of antisense target knockdown with a MOE gapmer antisense oligonucleotide was reported by Kastelein and colleagues (Ref. 1). Following on from animal studies (Ref. 101; discussed earlier in this section), these workers reported that simple intravenous administration of an antisense oligonucleotide targeting apolipoprotein B mRNA in humans produced dose-dependent reductions in circulating apolipoprotein B protein levels and LDL–cholesterol levels. Although not a direct measure of antisense target mRNA knockdown, the ability to measure changes in circulating levels of apolipoprotein B protein, and the clear dose-dependency of the protein knockdown and phenotype changes (reduced LDL–cholesterol) in this study provide convincing evidence of successful antisense targeted apolipoprotein B mRNA knockdown.

Conclusions and outstanding research questions

The large body of literature describing successful antisense use in vivo is ample evidence that this method of gene silencing is robust and reproducible. The antisense platform has enjoyed a considerable 'head-start' on other gene-silencing strategies such as those employing RNA interference. Lessons learnt from this period, in addition to those from extensive development of siRNA agents as therapeutics, provide great hope that antisense drugs will emerge in the near term. In several cases, the nature and location of the target cell type and target gene product will allow the use of simple injection of antisense oligonucleotides as potent and safe therapeutic agents; this may well be the case for ISIS 301012. For the liver, renal proximal tubules and the spleen, simple systemic administration may be sufficient to produce safe and effective therapeutic gene silencing. Where other cell types are targeted, delivery systems that overcome the 'trapping' of oligonucleotide within the liver, kidney and spleen and resultant low tissue concentrations are very likely to improve therapeutic indices. While in some instances this has already been achieved using liposomal delivery, endosomal escape agents, and targeting moieties, a step-change in antisense oligonucleotide therapeutics calls for further progress in antisense delivery systems.

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References

1 Kastelein, J.J.P. et al. (2006) Potent reduction of apolipoprotein B and low-density lipoprotein cholesterol by short-term administration of an antisense inhibitor of apolipoprotein B. Circulation 114, 1729-1735
2 Lemonidis, K.M. et al. (2006) Pharmacological profiling of apolipoprotein B-100 and microsomal triglyceride transfer protein antisense inhibitors in hyperlipidemic mice. Arteriosclerosis, Thrombosis, and Vascular Biology 26, E60-E61
3 Wang, Y. (2007) Isis-301012 - Antisense inhibitor of apolipoprotein B treatment of lipoprotein disorders. Drugs of the Future 32, 699-702
4 Aartsma-Rus, A. et al. (2004) Comparative analysis of antisense oligonucleotide analogs for targeted DMD exon 46 skipping in muscle cells. Gene Therapy 11, 1391-1398
5 Alter, J. et al. (2006) Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology. Nature Medicine 12, 175-177
6 Arechavala, V. et al. (2006) A phase I/II clinical trial in Duchenne muscular dystrophy using IM and IV delivered antisense oligonucleotides: The MDEX consortium. Neuromuscular Disorders 16, 685
7 McHutchison, J.G. et al. (2006) A phase I trial of an antisense inhibitor of hepatitis C virus (ISIS 14803), administered to chronic hepatitis C patients. Journal of Hepatology 44, 88-96
8 Devi, G.R. et al. (2005) In vivo bioavailability and pharmacokinetics of a c-MYC antisense phosphorodiamidate morpholino oligomer, AVI-4126, in solid tumors. Clinical Cancer Research 11, 3930-3938
9 Iversen, P.L. et al. (2003) Efficacy of antisense morpholino oligomer targeted to c-myc in prostate cancer xenograft murine model and a phase I safety study in humans. Clinical Cancer Research 9, 2510-2519
10 Leonetti, C. et al. (1996) Antitumor effect of c-myc antisense phosphorothioate oligodeoxynucleotides on human melanoma cells in vitro and and in mice. Journal of the National Cancer Institute 88, 419-429

Accession information: doi:10.1017/S1462399409001021; Vol. 11; e10; March 2009
© 2009 Cambridge University Press

11 Crooke, S.T. (2004) Progress in antisense technology. Annual Review of Medicine 55, 61-95
12 Galderisi, U., Cascino, A. and Giordano, A. (1999) Antisense oligonucleotides as therapeutic agents. Journal of Cellular Physiology 181, 251-257
13 Kastelein, J.P. et al. (2007) ISIS 301012, an antisense inhibitor of APOB, produces significant additional reduction of LDL-C & APOB in hypercholesterolemic subjects on statins not meeting target. Atherosclerosis Supplements 8, 198-199
14 Yin, H., Lu, Q. and Wood, M. (2008) Effective exon skipping and restoration of dystrophin expression by peptide nucleic acid antisense oligonucleotides in mdx mice. Molecular Therapy 16, 38-45
15 Madsen, E.C. et al. (2008) In vivo correction of a Menkes disease model using antisense oligonucleotides. Proceedings of the National Academy of Sciences of the United States of America 105, 3909-3914
16 Henke, E. et al. (2008) Peptide-conjugated antisense oligonucleotides for targeted inhibition of a transcriptional regulator in vivo. Nature Biotechnology 26, 91-100
17 Urban, E. and Noe, C.R. (2003) Structural modifications of antisense oligonucleotides. Farmaco 58, 243-258
18 Kaur, H., Babu, B.R. and Maiti, S. (2007) Perspectives on chemistry and therapeutic applications of Locked Nucleic Acid (LNA). Chemical Reviews 107, 4672-4697
19 Faria, M. and Ulrich, H. (2008) Sugar boost: when ribose modifications improve oligonucleotide performance. Current Opinion in Molecular Therapeutics 10, 168-175
20 Prakash, T.P. and Bhat, B. (2007) 2′-Modified oligonucleotides for antisense therapeutics. Current Topics in Medicinal Chemistry 7, 641-649
21 Crooke, S., ed. (2008) Antisense Drug Technology; Principles, Strategies and Applications, CRC Press, Boca Raton, FL, USA
22 Iversen, P.L. (2001) Phosphorodiamidate morpholino oligomers: favorable properties for sequence-specific gene inactivation. Current Opinion in Molecular Therapeutics 3, 235-238
23 Nielsen, E. (2006) RNA targeting using peptide nucleic acid. Handbook of Experimental Pharmacology 395-403
24 Jepsen, J.S., Sorensen, M.D. and Wengel, J. (2004) Locked nucleic acid: a potent nucleic acid analog in therapeutics and biotechnology. Oligonucleotides 14, 130-146
25 Monia, B.P. et al. (1993) Evaluation of 2′-modified oligonucleotides containing 2′-deoxy gaps as antisense inhibitors of gene expression. Journal of Biological Chemistry 268, 14514-14522
26 Fluiter, K. et al. (2005) On the in vitro and in vivo properties of four locked nucleic acid nucleotides incorporated into an anti-H-Ras antisense oligonucleotide. Chembiochem 6, 1104-1109
27 Grunweller, A. et al. (2003) Comparison of different antisense strategies in mammalian cells using locked nucleic acids, 2′-O-methyl RNA, phosphorothioates and small interfering RNA. Nucleic Acids Research 31, 3185-3193
28 Monia, B.P. et al. (1993) Evaluation of 2′-modified oligonucleotides containing 2′-deoxy gaps as antisense inhibitors of gene expression. Journal of Biological Chemistry 268, 14514-14522
29 Yu, R.Z. et al. (2007) Cross-species pharmacokinetic comparison from mouse to man of a second-generation antisense oligonucleotide, ISIS 301012, targeting human apolipoprotein B-100. Drug Metabolism and Disposition 35, 460-468
30 McMahon, B.M. et al. (2002) Pharmacokinetics and tissue distribution of a peptide nucleic acid after intravenous administration. Antisense and Nucleic Acid Drug Development 12, 65-70
31 Yu, R.Z. et al. (2004) Tissue disposition of 2′-O-(2-methoxy) ethyl modified antisense oligonucleotides in monkeys. Journal of Pharmaceutical Sciences 93, 48-59
32 Raoof, A.A. et al. (2004) Oral bioavailability and multiple dose tolerability of an antisense oligonucleotide tablet formulated with sodium caprate. Journal of Pharmaceutical Sciences 93, 1431-1439
33 Zhang, R. et al. (1995) Pharmacokinetics and tissue distribution in rats of an oligodeoxynucleotide phosphorothioate (GEM 91) developed as a therapeutic agent for human immunodeficiency virus type-1. Biochemical Pharmacology 49, 929-939
34 Sewell, K.L. et al. (2002) Phase I trial of ISIS 104838, a 2′-methoxyethyl modified antisense oligonucleotide targeting tumor necrosis factor-alpha. Journal of Pharmacology and Experimental Therapeutics 303, 1334-1343
35 Marcucci, G. et al. (2003) Phase 1 and pharmacodynamic studies of G3139, a Bcl-2 antisense oligonucleotide, in combination with chemotherapy in refractory or relapsed acute leukemia. Blood 101, 425-432
36 Cossum, P.A. et al. (1994) Pharmacokinetics of a 14C-labeled phosphorothioate oligonucleotide,

Accession information: doi:10.1017/S1462399409001021; Vol. 11; e10; March 2009
© 2009 Cambridge University Press

ISIS 2105, after intradermal administration to rats. Journal of Pharmacology and Experimental Therapeutics 269, 89-94

37 Barbee, R.W. et al. (1992) Microsphere and dilution techniques for the determination of blood flows and volumes in conscious mice. American Journal of Physiology 263, R728-733

38 Delp, M.D. et al. (1991) Distribution of cardiac output during diurnal changes of activity in rats. American Journal of Physiology 261, H1487-1493

39 Geary, R.S. et al. (1997) Pharmacokinetics and metabolism in mice of a phosphorothioate oligonucleotide antisense inhibitor of C-raf-1 kinase expression. Drug Metabolism and Disposition 25, 1272-1281

40 Geary, R.S. et al. (2003) Pharmacokinetics of a tumor necrosis factor-alpha phosphorothioate 2′-O-(2-methoxyethyl) modified antisense oligonucleotide: Comparison across species. Drug Metabolism and Disposition 31, 1419-1428

41 Rayburn, E.R. and Zhang, R. (2008) Antisense, RNAi, and gene silencing strategies for therapy: mission possible or impossible? Drug Discovery Today 13, 513-521

42 AEGIS (1997) Hybridon stops development of Gem(R)91 for AIDS; resources to be focused on four 2nd generation compounds, http://www.aegis.com/news/pr/1997/PR970720.html

43 Roivainen, A. et al. (2004) 68 Ga-labeled oligonucleotides for in vivo imaging with PET. Journal of Nuclear Medicine 45, 347-355

44 Lendvai, G. et al. (2005) Biodistribution of 68 Ga-labelled phosphodiester, phosphorothioate, and 2′-O-methyl phosphodiester oligonucleotides in normal rats. European Journal of Pharmaceutical Sciences 26, 26-38

45 Kuhnast, B. et al. (2000) General method to label antisense oligonucleotides with radioactive halogens for pharmacological and imaging studies. Bioconjugate Chemistry 11, 627-636

46 Fluiter, K. et al. (2003) In vivo tumor growth inhibition and biodistribution studies of locked nucleic acid (LNA) antisense oligonucleotides. Nucleic Acids Research 31, 953-962

47 Agrawal, S. et al. (1997) Mixed-backbone oligonucleotides as second generation antisense oligonucleotides: in vitro and in vivo studies. Proceedings of the National Academy of Sciences of the United States of America 94, 2620-2625

48 Amantana, A. et al. (2007) Pharmacokinetics, biodistribution, stability and toxicity of a cell-penetrating peptide-morpholino oligomer conjugate. Bioconjugate Chemistry 18, 1325-1331

49 Sawai, K. et al. (1996) Disposition of oligonucleotides in isolated perfused rat kidney: involvement of scavenger receptors in their renal uptake. Journal of Pharmacology and Experimental Therapeutics 279, 284-290

50 Geary, R.S. et al. (2001) Pharmacokinetic properties of 2′-O-(2-methoxyethyl)-modified oligonucleotide analogs in rats. Journal of Pharmacology and Experimental Therapeutics 296, 890-897

51 Sazani, P. et al. (2002) Systemically delivered antisense oligomers upregulate gene expression in mouse tissues. Nature Biotechnology 20, 1228-1233

52 Rappaport, J. et al. (1995) Transport of phosphorothioate oligonucleotides in kidney: implications for molecular therapy. Kidney International 47, 1462-1469

53 Oberbauer, R., Schreiner, G.F. and Meyer, T.W. (1995) Renal uptake of an 18-mer phosphorothioate oligonucleotide. Kidney International 48, 1226-1232

54 Masarjian, L. et al. (2004) Distribution and excretion of a phosphorothioate oligonucleotide in rats with experimentally induced renal injury. Oligonucleotides 14, 299-310

55 Monteith, D.K. et al. (1999) Evaluation of the renal effects of an antisense phosphorothioate oligodeoxynucleotide in monkeys. Toxicologic Pathology 27, 307-317

56 Sawai, K. et al. (1995) Renal disposition characteristics of oligonucleotides modified at terminal linkages in the perfused rat kidney. Antisense Research and Development 5, 279-287

57 Steward, A. et al. (1998) Co-administration of polyanions with a phosphorothioate oligodeoxynucleotide (CGP 69846A): a role for the scavenger receptor in its in vivo disposition. Biochemical Pharmacology 56, 509-516

58 Lendvai, G. et al. (2008) Biodistribution of 68 Ga-labeled LNA-DNA mixmer antisense oligonucleotides for rat chromogranin-A. Oligonucleotides 18, 33-49

59 Wallace, T.L. et al. (1997) Pharmacokinetics and distribution of a 33P-labeled anti-human immunodeficiency virus oligonucleotide (AR177) after single- and multiple-dose intravenous administration to rats. Journal of Pharmacology and Experimental Therapeutics 280, 1480-1488

60 Butler, M., Stecker, K. and Bennett, C.F. (1997) Cellular distribution of phosphorothioate oligodeoxynucleotides in normal rodent tissues. Laboratory Investigation 77, 379-388

61 Graham, M.J. et al. (1998) In vivo distribution and metabolism of a phosphorothioate oligonucleotide

Accession information: doi:10.1017/S1462399409001021; Vol. 11; e10; March 2009
© 2009 Cambridge University Press

within rat liver after intravenous administration. Journal of Pharmacology and Experimental Therapeutics 286, 447-458

62 Butler, M. et al. (2000) Phosphorothioate oligodeoxynucleotides distribute similarly in class A scavenger receptor knockout and wild-type mice. Journal of Pharmacology and Experimental Therapeutics 292, 489-496

63 Bijsterbosch, M.K. et al. (2002) bis-Cholesteryl-conjugated phosphorothioate oligodeoxynucleotides are highly selectively taken up by the liver. Journal of Pharmacology and Experimental Therapeutics 302, 619-626

64 Yu, R.Z. et al. (1999) Pharmacokinetics and tissue disposition in monkeys of an antisense oligonucleotide inhibitor of Ha-ras encapsulated in stealth liposomes. Pharmaceutical Research 16, 1309-1315

65 Ponnappa, B.C. et al. (2005) Inhibition of tumor necrosis factor alpha secretion and prevention of liver injury in ethanol-fed rats by antisense oligonucleotides. Biochemical Pharmacology 69, 569-577

66 Tyler, B.M. et al. (1999) Peptide nucleic acids targeted to the neurotensin receptor and administered i.p. cross the blood-brain barrier and specifically reduce gene expression. Proceedings of the National Academy of Sciences of the United States of America 96, 7053-7058

67 Greish, K. et al. (2003) Macromolecular therapeutics: advantages and prospects with special emphasis on solid tumour targeting. Clinical Pharmacokinetics 42, 1089-1105

68 White, P.J. et al. (1999) Live confocal microscopy of oligonucleotide uptake by keratinocytes in human skin grafts on nude mice. Journal of Investigative Dermatology 112, 887-892

69 White, P.J. et al. (1999) Oligonucleotide uptake in cultured keratinocytes: influence of confluence, cationic liposomes, and keratinocyte cell type. Journal of Investigative Dermatology 112, 699-705

70 Dick, J.M. et al. (2001) Antisense knockdown of inducible nitric oxide synthase inhibits the relaxant effect of VIP in isolated smooth muscle cells of the mouse gastric fundus. British Journal of Pharmacology 134, 425-433

71 Roth, C.M. (2005) Molecular and cellular barriers limiting the effectiveness of antisense oligonucleotides. Biophysical Journal 89, 2286-2295

72 Roberts, J. et al. (2006) Efficient and persistent splice switching by systemically delivered LNA oligonucleotides in mice. Molecular Therapy 14, 471-475

73 Astriab-Fisher, A. et al. (2002) Conjugates of antisense oligonucleotides with the Tat and antennapedia cell-penetrating peptides: effects on cellular uptake, binding to target sequences, and biologic actions. Pharmaceutical Research 19, 744-754

74 Astriab-Fisher, A. et al. (2000) Antisense inhibition of P-glycoprotein expression using peptide-oligonucleotide conjugates. Biochemical Pharmacology 60, 83-90

75 Juliano, R. et al. (2008) Mechanisms and strategies for effective delivery of antisense and siRNA oligonucleotides. Nucleic Acids Research 36, 4158-4171

76 Seong, J.H. et al. (2006) Polyethylenimine-based antisense oligodeoxynucleotides of IL-4 suppress the production of IL-4 in a murine model of airway inflammation. Journal of Gene Medicine 8, 314-323

77 Fischer, D. et al. (2004) Effect of poly(ethylene imine) molecular weight and pegylation on organ distribution and pharmacokinetics of polyplexes with oligodeoxynucleotides in mice. Drug Metabolism and Disposition 32, 983-992

78 Kilic, A.C. et al. (2005) Preparation and characterization of PLGA nanospheres for the targeted delivery of NR2B-specific antisense oligonucleotides to the NMDA receptors in the brain. Journal of Microencapsulation 22, 633-641

79 Springate, C.M. et al. (2008) Clusterin antisense complexed with chitosan for controlled intratumoral delivery. International Journal of Pharmaceutics 350, 53-64

80 Boyd, B.J. et al. (2006) Cationic poly-L-lysine dendrimers: pharmacokinetics, biodistribution, and evidence for metabolism and bioresorption after intravenous administration to rats. Molecular Pharmaceutics 3, 614-627

81 Yoo, H., Sazani, P. and Juliano, R.L. (1999) PAMAM dendrimers as delivery agents for antisense oligonucleotides. Pharmaceutical Research 16, 1799-1804

82 Kaminskas, L.M. et al. (2008) The impact of molecular weight and PEG chain length on the systemic pharmacokinetics of PEGylated poly l-lysine dendrimers. Molecular Pharmaceutics 5, 449-463

83 Dash, P.R. et al. (1999) Factors affecting blood clearance and in vivo distribution of polyelectrolyte complexes for gene delivery. Gene Therapy 6, 643-650

Accession information: doi:10.1017/S1462399409001021; Vol. 11; e10; March 2009
© 2009 Cambridge University Press

84 Merdan, T. et al. (2005) PEGylation of poly(ethylene imine) affects stability of complexes with plasmid DNA under in vivo conditions in a dose-dependent manner after intravenous injection into mice. Bioconjugate Chemistry 16, 785-792
85 Ward, C.M., Read, M.L. and Seymour, L.W. (2001) Systemic circulation of poly(L-lysine)/DNA vectors is influenced by polycation molecular weight and type of DNA: differential circulation in mice and rats and the implications for human gene therapy. Blood 97, 2221-2229
86 Anwer, K. et al. (2000) Cationic lipid-based delivery system for systemic cancer gene therapy. Cancer Gene Therapy 7, 1156-1164
87 Hofland, H.E. et al. (1997) In vivo gene transfer by intravenous administration of stable cationic lipid/DNA complex. Pharmaceutical Research 14, 742-749
88 Liu, F. et al. (1997) Factors controlling the efficiency of cationic lipid-mediated transfection in vivo via intravenous administration. Gene Therapy 4, 517-523
89 Mahato, R.I. et al. (1998) Biodistribution and gene expression of lipid/plasmid complexes after systemic administration. Human Gene Therapy 9, 2083-2099
90 Szoka, F. (2008) Molecular biology. The art of assembly. Science 319, 578-579
91 Li, W. and Szoka, F.C., Jr. (2007) Lipid-based nanoparticles for nucleic acid delivery. Pharmaceutical Research 24, 438-449
92 Peer, D. et al. (2008) Systemic leukocyte-directed siRNA delivery revealing cyclin D1 as an anti-inflammatory target. Science 319, 627-630
93 Rozema, D.B. et al. (2007) Dynamic PolyConjugates for targeted in vivo delivery of siRNA to hepatocytes. Proceedings of the National Academy of Sciences of the United States of America 104, 12982-12987
94 Wolff, J.A. and Rozema, D.B. (2008) Breaking the bonds: non-viral vectors become chemically dynamic. Molecular Therapy 16, 8-15
95 Stein, C.A. and Krieg, A.M. (1994) Problems in interpretation of data derived from in vitro and in vivo use of antisense oligodeoxynucleotides. Antisense Research and Development 4, 67-69
96 Nguyen, T.T. et al. (2006) Intravenous insulin-like growth factor-I receptor antisense treatment reduces angiotensin receptor expression and function in spontaneously hypertensive rats. Journal of Pharmacology and Experimental Therapeutics 318, 1171-1177
97 Nguyen, T.T. and White, P.J. (2005) Intravenous IGF-I receptor antisense reduces IGF-IR expression and diminishes pressor responses to angiotensin II in conscious normotensive rats. British Journal of Pharmacology 146, 935-941
98 Kagiyama, S. et al. (2003) Antisense to epidermal growth factor receptor prevents the development of left ventricular hypertrophy. Hypertension 41, 824-829
99 Zhang, Y.C. et al. (2000) Antisense inhibition of beta(1)-adrenergic receptor mRNA in a single dose produces a profound and prolonged reduction in high blood pressure in spontaneously hypertensive rats. Circulation 101, 682-688
100 Lu, Q.L. et al. (2005) Systemic delivery of antisense oligoribonucleotide restores dystrophin expression in body-wide skeletal muscles. Proceedings of the National Academy of Sciences of the United States of America 102, 198-203
101 Crooke, R.M. et al. (2005) An apolipoprotein B antisense oligonucleotide lowers LDL cholesterol in hyperlipidemic mice without causing hepatic steatosis. Journal of Lipid Research 46, 872-884
102 Guha, M. et al. (2007) Specific down-regulation of connective tissue growth factor attenuates progression of nephropathy in mouse models of type 1 and type 2 diabetes. The FASEB Journal 21, 3355-3368
103 Tyler, B.M. et al. (1999) Peptide nucleic acids targeted to the neurotensin receptor and administered i.p. cross the blood-brain barrier and specifically reduce gene expression. Proceedings of the National Academy of Sciences of the United States of America 96, 7053-7058
104 Lu, Q.L. et al. (2003) Functional amounts of dystrophin produced by skipping the mutated exon in the mdx dystrophic mouse. Nature Medicine 9, 1009-1014
105 Alter, J. et al. (2006) Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology. Nature Medicine 12, 175-177
106 Yin, H.F., Lu, Q.L. and Wood, M. (2008) Effective exon skipping and restoration of dystrophin expression by peptide nucleic acid antisense oligonucleotides in mdx mice. Molecular Therapy 16, 38-45
107 Reich, S.J. et al. (2003) Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. Molecular Vision 9, 210-216
108 Xing, H.R. et al. (2003) Pharmacologic inactivation of kinase suppressor of ras-1 abrogates Ras-mediated pancreatic cancer. Nature Medicine 9, 1266-1268

Accession information: doi:10.1017/S1462399409001021; Vol. 11; e10; March 2009
© 2009 Cambridge University Press

109 Sazani, P. et al. (2001) Nuclear antisense effects of neutral, anionic and cationic oligonucleotide analogs. Nucleic Acids Research 29, 3965-3974
110 Cao, N. et al. (2006) Characterization of the acute cardiovascular effects of intravenously administered insulin-like growth factor-I in conscious Sprague-Dawley rats. Clinical and Experimental Pharmacology and Physiology 33, 1190-1195
111 Myers, K.J. et al. (2005) Antisense oligonucleotide blockade of alpha 4 integrin prevents and reverses clinical symptoms in murine experimental autoimmune encephalomyelitis. Journal of Neuroimmunology 160, 12-24
112 Lau, Q.C. et al. (2002) In vivo pro-apoptotic and antitumor efficacy of a c-Raf antisense phosphorothioate oligonucleotide: relationship to tumor size. Antisense and Nucleic Acid Drug Development 12, 11-20
113 Xing, H.R. et al. (2003) Pharmacologic inactivation of kinase suppressor of ras-1 abrogates Ras-mediated pancreatic cancer. Nature Medicine 9, 1266-1268
114 Kastelein, J.J. et al. (2006) Potent reduction of apolipoprotein B and low-density lipoprotein cholesterol by short-term administration of an antisense inhibitor of apolipoprotein B. Circulation 114, 1729-1735
115 van Deventer, S.J., Tami, J.A. and Wedel, M.K. (2004) A randomised, controlled, double blind, escalating dose study of alicaforsen enema in active ulcerative colitis. Gut 53, 1646-1651
116 Group, V.S. (2002) A randomized controlled clinical trial of intravitreous fomivirsen for treatment of newly diagnosed peripheral cytomegalovirus retinitis in patients with AIDS. American Journal of Ophthalmology 133, 467-474
117 Cunningham, C.C. et al. (2001) A Phase I trial of H-ras antisense oligonucleotide ISIS 2503 administered as a continuous intravenous infusion in patients with advanced carcinoma. Cancer 92, 1265-1271
118 Cunningham, C.C. et al. (2000) A phase I trial of c-Raf kinase antisense oligonucleotide ISIS 5132 administered as a continuous intravenous infusion in patients with advanced cancer. Clinical Cancer Research 6, 1626-1631
119 Marshall, J. et al. (2004) A phase I trial of a Bcl-2 antisense (G3139) and weekly docetaxel in patients with advanced breast cancer and other solid tumors. Annals of Oncology 15, 1274-1283
120 Adjei, A.A. et al. (2003) A phase I trial of ISIS 2503, an antisense inhibitor of H-ras, in combination with gemcitabine in patients with advanced cancer. Clinical Cancer Research 9, 115-123
121 Mani, S. et al. (2002) Phase I clinical and pharmacokinetic study of protein kinase C-alpha antisense oligonucleotide ISIS 3521 administered in combination with 5-fluorouracil and leucovorin in patients with advanced cancer. Clinical Cancer Research 8, 1042-1048
122 Stevenson, J.P. et al. (1999) Phase I clinical/pharmacokinetic and pharmacodynamic trial of the c-raf-1 antisense oligonucleotide ISIS 5132 (CGP 69846A). Journal of Clinical Oncology 17, 2227-2236
123 Bishop, M.R. et al. (1996) Phase I trial of an antisense oligonucleotide OL(1)p53 in hematologic malignancies. Journal of Clinical Oncology 14, 1320-1326
124 Bedikian, A.Y. et al. (2006) Bcl-2 antisense (oblimersen sodium) plus dacarbazine in patients with advanced melanoma: the Oblimersen Melanoma Study Group. Journal of Clinical Oncology 24, 4738-4745
125 O'Brien, S. et al. (2007) Randomized phase III trial of fludarabine plus cyclophosphamide with or without oblimersen sodium (Bcl-2 antisense) in patients with relapsed or refractory chronic lymphocytic leukemia. Journal of Clinical Oncology 25, 1114-1120
126 Bijsterbosch, M.K. et al. (2002) bis-Cholesteryl-conjugated phosphorothioate oligodeoxynucleotides are highly selectively taken up by the liver. Journal of Pharmacology and Experimental Therapeutics 302, 619-626
127 Sun, Y. et al. (2006) Suppression of tumor growth using antisense oligonucleotide against survivin in an orthotopic transplant model of human hepatocellular carcinoma in nude mice. Oligonucleotides 16, 365-374

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Further reading, resources and contacts

Publications

Crooke, S., ed. (2008) Antisense Drug Technology; Principles, Strategies and Applications, CRC Press, Boca Raton, FL, USA

This book provides a comprehensive review of the development of antisense oligonucleotides as therapeutic agents. The chapter authors are from ISIS pharmaceuticals, which perhaps limits the discussion of antisense oligonucleotide chemistries other than MOE, but this book remains indispensable to those in the field of antisense therapeutics.

Rayburn, E.R. and Zhang, R. (2008) Antisense, RNAi, and gene silencing strategies for therapy: mission possible or impossible? Drug Discovery Today 13, 513-521

This article includes an up-to-date listing of past and current clinical trials using antisense oligonucleotides.

Juliano, R. et al. (2008) Mechanisms and strategies for effective delivery of antisense and siRNA oligonucleotides. Nucleic Acids Research 36, 4158-4171

This review provides an insightful look at overcoming the barriers to silencing for both single- and double-stranded oligonucleotides, from a group with extensive experience in the field.

Websites

The RNAi.net website contains breaking news stories, job vacancies, product information and conference information in the field of RNAi:

http://www.rnai.net/

Features associated with this article

Figures

Figure 1. Fates of antisense oligonucleotides following systemic administration.
Figure 2. Schematic representation of a colloidal particle for antisense delivery.

Tables

Table 1. Tissue distribution of the major classes of modified antisense oligonucleotides after systemic administration.
Table 2. Barriers faced by antisense oligonucleotides when given systemically, and approaches for overcoming them.
Table 3. Representative examples of the determination of maximal efficacy of in vivo knockdown.

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