Developing antisense oligonucleotides from the laboratory to clinical trials

David E. Szymkowski

Antisense gene therapy has attracted the attention of the pharmaceutical industry with its promise of simple and specific rational drug design. These 'informational drugs' uniquely target gene expression, unlike traditional therapeutics, which block activity of an existing protein. Oligonucleotide therapies are now developing a strong proof-of-concept foundation based on *in vitro* and animal models, and several clinical trials in progress aim to demonstrate that these compounds can be safe and effective drugs. With pharmacodynamic, toxicological and efficacy data accumulating from animal and human studies, the critical goals are to enhance compound delivery and stability, and reduce the nonspecific toxic effects of these rational therapeutics.

In theory, antisense oligodeoxynucleotides (ODNs) fulfill practically every requirement of rational drug design. Using the simple and readily applied structure–activity relationship of Watson–Crick base pairing, compounds with excellent target specificity can be identified from a primary gene sequence and tested in a variety of established assays. Active compounds can be generated and evaluated in the absence of structural information about the ultimate protein target. Pharmacodynamic properties of ODNs are often a consequence of backbone structure[1], and ODNs with numerous sugar and phosphate modifications have demonstrated good safety profiles in animal and human studies[2–4], suggesting that new sequences with identical backbones may have similar properties. In spite of these apparent advantages, hopes that the oligonucleotide approach could rapidly complement traditional drug discovery have been tempered by problems in developing a new and unfamiliar class of molecules for unconventional targets. Nonspecific effects have often made it difficult to interpret biological activity, yet even non-antisense ODNs have been exploited as potential drugs. This review focuses on oligonucleotide drug candidates that are in, or near to entering, human trials, and attempts to address the critical pharmacological and toxicological issues to be resolved before this intriguing rational approach to drug discovery can generate effective, safe and economical new therapeutics.

Oligonucleotide mechanisms of action

Soon after the structure and function of nucleic acids were elucidated in the 1950s and 1960s, researchers suggested that oligonucleotides could interact with DNA and RNA to modulate their metabolism (for a historical review, see Zon[5]). Miller and Ts'O at Johns Hopkins[6] and Zamecnik and Stephens at Harvard first demonstrated that ODNs could produce sequence-specific biological effects. Zamecnik's group inhibited replication of Rous sarcoma virus *in vitro* to demonstrate an antisense effect in a biological system[7]. With the introduction of routine DNA sequencing and automated oligonucleotide synthesis in the early 1980s, antisense publications have grown from a handful before 1986 to many

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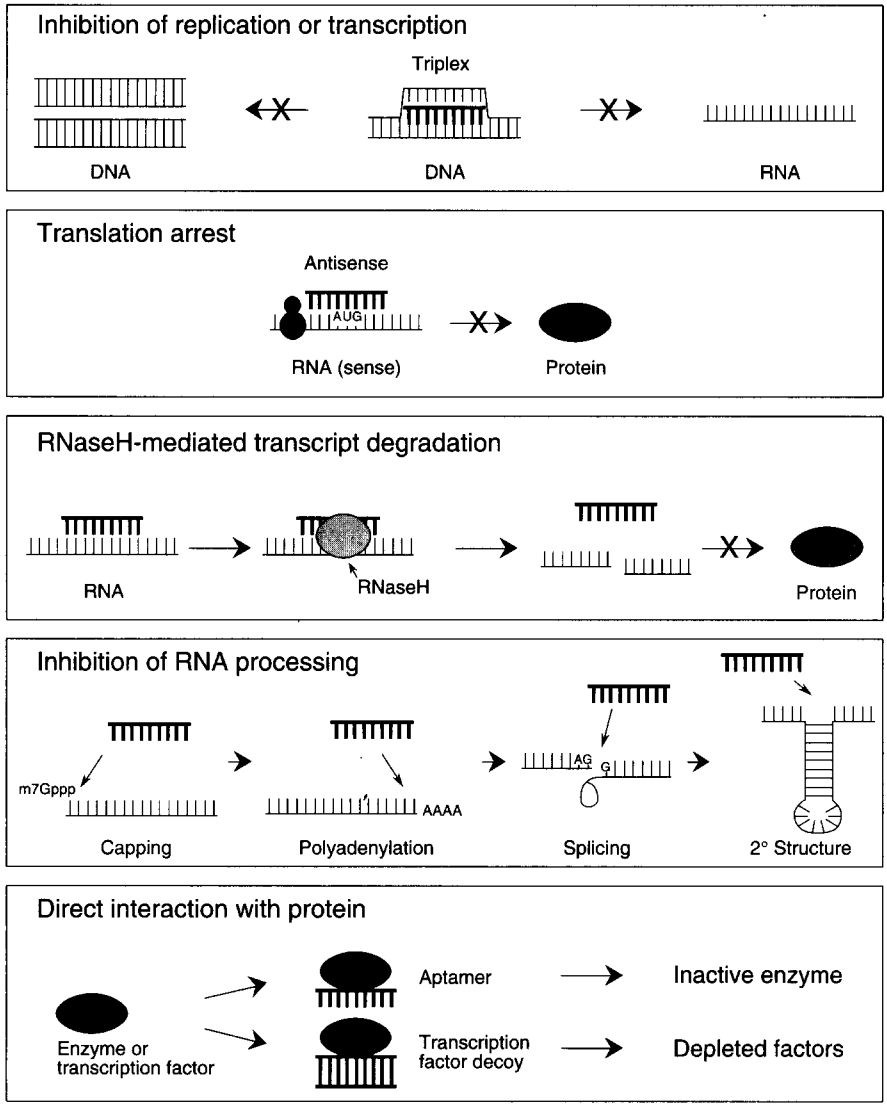

Figure 1. *Potential mechanisms of action for therapeutic oligonucleotides. Antisense molecules may sterically inhibit replication, transcription or translation, recruit RNase H to target RNA, or block RNA processing. Aptamer oligonucleotides bind proteins directly through a specific sequence or secondary structure. Duplex ODNs may sequester proteins, including transcription factors, and interfere with gene regulation.*

thousands in the past ten years. Much of this research has been generated by biotechnology companies collaborating with the established pharmaceutical industry, and numerous patents have been filed to protect ODN sequence, structure and formulation. At least twelve compounds have been approved for clinical trials, and it is likely that more than one oligonucleotide drug will reach the market in the next five to ten years.

Antisense therapy relies on the hybridization of an oligodeoxynucleotide to a 'sense' RNA transcript in a sequence-specific manner to block production of the target protein. It can be difficult to prove, especially *in vivo*, that a given biological effect produced by an ODN results from this sequence-specific reduction of gene expression[8]; therefore, the general term 'oligonucleotide therapeutic' encompasses variations of the original antisense concept including those that exploit sequence- or target-nonspecific activities. Oligonucleotides can interact with a gene, a nascent RNA transcript or its processing, or even a protein product (Figure 1)[9,10]. Non-antisense effects, such as triplex formation (through Hoogsteen base pairing)[11] and transcription factor decoy approaches[12,13], have been exploited to extend the possible areas of therapeutic intervention for ODNs beyond interaction with mRNA. Protein-binding oligonucleotides known as aptamers[14] can be identified from combinatorial libraries, and are similar in concept to small-molecule enzyme inhibitors. Mechanisms by which antisense ODNs reduce protein production include inhibition of translation and directed degradation of the RNA target[4]. Oligonucleotide structures with high affinity for RNA should be most effective at inhibiting protein production by blocking ribosome translocation. Alternatively, common charged ODNs containing phosphodiester (PO) or phosphorothioate (PS) backbones (Figure 2) can stimulate degradation of a transcript by RNase H, a nuclear enzyme that cleaves the RNA strand of a DNA:RNA duplex. If RNase H is involved in cellular activity, as recent evidence suggests[15], then antisense ODNs may be unique drugs with the potential to destroy an RNA target catalytically. ODNs may interfere with gene expression at many other points (Figure 1), but precise mechanisms of action are not easily

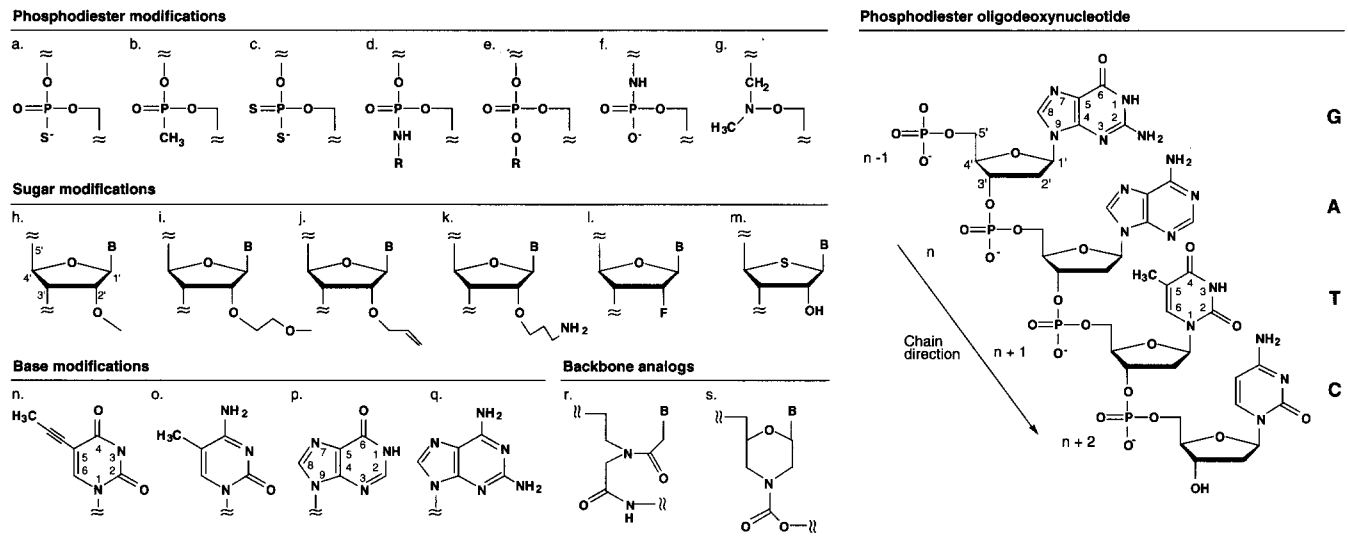

Figure 2. *Examples of structural modifications to ODNs[67,99–101]. The normal phosphodiester deoxyribonucleotide backbone (right) has been altered to: (a) phosphorothioate; (b) methylphosphonate; (c) phosphorodithioate; (d) phosphoramidates; (e) phosphotriesters; (f)* N3′–P5′ *phosphoramidate; and (g) methylene methylimino (compounds d and e permit further changes at the R position). Deoxyribose modifications include: alkylation at the 2′ position (B = base) [(h) 2′-methoxyribose; (i) 2′-methoxyethoxyribose; (j) 2′-O-allyl]; (k) 2′-aminopropoxy; (l) 2′-fluororibose; and (m) ring oxygen substitution to a thiohexose analog[102]. Base modifications include: (n) C5-propynyl uracil; (o) 5-methylcytosine; (p) inosine (used as a wobble base for ambiguous target sequences); and (q) 2-aminoadenosine. Backbone analogs include (r) peptide nucleic acid (PNA)[71]; and (s) a methylcarbamate polymer (B = base).*

defined within the cell or *in vivo*, and may vary for different sequences and structures.

Antisense versus traditional drug development

Antisense methods have several potential advantages over conventional drug design. In contrast to high-throughput screening or molecular modeling, the primary advantage of antisense drug discovery is its rapid generation of initial leads. Active leads may often be identified from a small number of compounds, compared with the thousands typically screened to identify 'hits' in other drug discovery programs. Target-gene identification and sequencing is sufficient for initial drug design and screening; therefore, structural analyses of RNA or protein currently contribute little to compound development. Finally, because ODNs share common characteristics, accelerated development of new molecules based on results with other compounds may be possible.

In spite of these theoretical advantages, the intrinsic properties of oligonucleotides have limited immediate successes *in vivo*. Phosphodiesters are rapidly degraded both extra- and intracellularly by ubiquitous nucleases, though recent advances in medicinal chemistry have substantially improved ODN stability[2]. The delivery of ODNs to the target cell *in vivo* has also been a significant obstacle to effective drug development. Furthermore, PS compounds demonstrate both sequence- and backbone-specific toxicity in animals, including lethal hypotensive effects in primates[16,17]. Finally, PS ODNs can induce sequence-nonspecific effects that may obscure an antisense component of biological activity[8,18]. However, the clear benefits of antisense technology have encouraged the pharmaceutical industry to attempt to overcome these barriers and develop effective oligonucleotide drugs.

Identifying a therapeutic target and sequence

Choosing a suitable gene

Careful experimental design is essential to identify antisense activity in appropriate assays, and to generate the toxicity, pharmacokinetic and efficacy data required to develop molecules for clinical trials. Antisense compounds can be proposed for any gene using only base-pairing rules. However, because of this simple structure–activity relationship, rigorous evidence must demonstrate that biological effects arise

Table 1. Clinical trials of oligonucleotides

Phase	Indication	Gene target	Compound	Company
III	Cytomegalovirus-induced retinitis in AIDS	MIE promoter of CMV	ISIS 2922	Isis/Eisai
II	Follicular lymphoma	*BCL-2*	G 1128, G 3139	Genta/Baxter Healthcare
II	Genital warts	E2 of HPV 6 & 11	ISIS 2105	Isis Pharmaceuticals
II	Rheumatoid arthritis, kidney transplant rejection, psoriasis, ulcerative colitis, Crohn's disease	*ICAM-1*	ISIS 2302	Isis/Boehringer Ingelheim
I/II	Acute and chronic myelogenous leukemia	c-*myb*	LR 3001	Lynx Therapeutics
I/II	AIDS	HIV *gag*	GEM 91	Hybridon
I/II	Chronic myelogenous leukemia	?	GT 1102	Genset
I	Acute myelogenous leukemia, advanced myelodysplastic syndrome	*p53*	OL(1)p53	Lynx Therapeutics
I	AIDS	HIV integrase (protein)	AR 177	Aronex Pharmaceuticals
I	AIDS	HIV *tat*	GPs 0193	Chugai Biopharmaceuticals
I	Restenosis	c-*myc*	4003W94	Lynx Therapeutics
I	Solid tumors	c-*raf* kinase	ISIS 5132	Isis/Ciba-Geigy
I	Solid tumors	*PKC-α*	ISIS 3521	Isis/Ciba-Geigy

from the anticipated reduction in target-gene expression[8]. Antisense development strategies for different therapeutic indications share common principles. First, a suitable RNA target must be identified for the selected disease. Viruses have been attractive targets because few effective drugs are available for common infections, and because viruses have small genomes with known essential genes, making it possible to identify relevant sequences. Viral sequences unrelated to the host genome can be chosen to reduce the chance of unexpected nonspecific effects, and antisense therapies can overcome drug resistance by modification of the base sequence to complement mutated viral strains. Finally, local delivery of ODNs at high concentration may be feasible for human papilloma virus (HPV), herpes simplex virus (HSV), cytomegalovirus (CMV) and other infections. Because of these advantages, five clinical trials have targeted viral diseases, including AIDS, AIDS-related CMV retinitis and genital warts (Table 1). Typical targets include viral DNA replication (E2 gene of HPV), early gene expression (in CMV) and viral core proteins (*gag* gene of HIV).

Cancers and inflammatory diseases often arise from aberrant gene expression in human cells, and present more challenging targets than infectious agents. To treat these diseases without interfering with normal cell metabolism, antisense activity must be aimed only at the abnormal gene in the affected cell. This specificity can be achieved by targeting oncogenes that are mutated or overexpressed in the abnormal cell[19]. Gene fusions such as *BCR–ABL* have been implicated in many cancers and are unique targets found only in malignant cells[20]. Target specificity can also be achieved through compound formulation and administration, including *ex vivo* treatments for leukemias[21], and by targeting the drug to a particular cell type using antibodies or receptor ligands[22].

Oligonucleotide selection

Once an appropriate disease and target gene have been chosen, an ODN sequence and structure that interacts with the RNA transcript must be identified. The phosphorothioate oligodeoxynucleotide (Figure 2a) has become the standard backbone for antisense research, because of its ease of synthesis and excellent nuclease stability relative to phosphodiesters[2]. Phosphorothioates have a slightly lower affinity for target RNA than do PO ODNs (about 0.5°C reduction in melting temperature per modification)[23] but a recent NMR study of an ODN:RNA duplex containing one PS linkage indicates that the nonbridging sulfur induces minimal alteration in the double helix (Figure 3)[24]. ODNs are usually designed in lengths from 17 to 25 nucleotides, based on a statistical evaluation of the length necessary to avoid random matches in the human genome or mRNA population, but even heptamers can show good activity and specificity in cells[25]. Typical targets on the RNA transcript include the 5′ CAP site, 5′ and 3′ untranslated regions, the translation-initiation site, internal intron/exon splice junctions and the

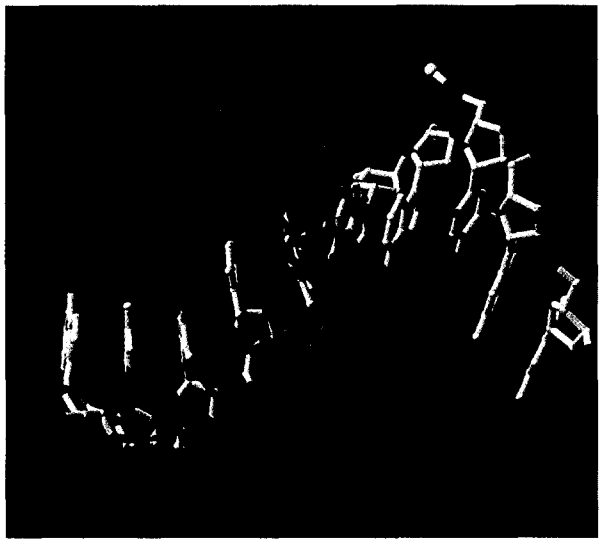

Figure 3. *NMR structure of the antisense DNA:RNA duplex d(GCTATAAsTGG)•r(CCAUUAUAGC) containing one phosphorothioate residue (indicated by the letter s in the sequence). Phosphorus is purple, oxygen is red, deoxyribonucleosides are white and ribonucleosides are blue. Sulfur (yellow) has minimal effect on base interactions or backbone configuration of either the* S *(shown) or* R *stereoisomers (Brookhaven reference 219d)*[24].

polyadenylation site[9] (Figure 1). However, antisense targets are not limited to these sites; RNA regions known or predicted to be single-stranded and therefore accessible to ODNs are also worth investigating[11].

Evaluation of biological efficacy

Cell-free assays

Observations of consistent antisense effects in several cell-free and *in vitro* assays (Box 1) substantiate claims that biological effects are caused by a reduction in expression of the targeted gene. An informative and widely used cell-free assay to identify active compounds is ODN-induced RNase H cleavage of a synthetic RNA transcript[15]. Selection of active sequences from random oligonucleotide libraries by similar RNase H assays can also identify accessible mRNA regions[26]. *In vitro* RNA-transcription and protein-translation assays[27] are also relevant measures of inhibition of gene expression. These and similar cell-free assays can provide the first indication that particular compounds interact with target RNA in a sequence-specific fashion and block its expression.

In vitro *assays*

New ODN structures are best evaluated in cell assays, where improvements in membrane permeability, intracellular stability, RNase H catalysis, toxicity and other relevant properties can be evaluated. In a virally infected or transformed cell, intracellular target-RNA or protein levels can be quantified

Box 1. Sensible antisense research

Proper design and interpretation of biological assays is especially important to identify sequence- and target-non-specific effects of ODNs[8]. Below are suggested assays and controls which can substantiate a true antisense mechanism of action.

Efficacy assays

- Cell-free assays: (1) hybrid ODN:RNA melting temperature, solubility and nuclease resistance of novel compounds[69]; (2) RNA degradation via ODN-catalyzed RNase H cleavage[15]; (3) RNA or protein reduction in *in vitro* transcription and translation extracts[27].
- Cell assays: (1) RNA reduction in cells by northern, ribonuclease protection or other analyses[30]; (2) protein reduction by western or enzyme activity[95]; (3) enzyme activity of transfected target-reporter gene fusions[44]; (4) inhibition of viral infection[14] or cell proliferation[96].
- Animal models: (1) direct RNA measurements as above[30]; (2) inhibition of viral infection[97]; (3) inhibition of tumor growth in nude mice[13,30,36].

Compound and target controls

- Mismatches: base changes in the compound and, if possible, target sequence, must reduce a biological effect. Mismatched gene targets (for example, different viral strains[98] or gene isotypes[30,31]) are particularly informative to evaluate non-antisense ODN effects.
- Random ODNs: a control containing four bases mixed at each position (a 20-nucleotide 'randomer' will consist of more than 10^{12} sequences) contains no informational content, and therefore is a powerful tool to distinguish sequence-specific from backbone-specific effects.
- Rearranged sequences: scrambled, sense and 'non-sense' (5′–3′ sequence, read as 3′–5′) ODNs eliminate target affinity, but can preserve base composition or secondary structure.
- Truncated sequences: below an optimal size, activity should inversely correlate with length. 'Shortmer' controls also identify the smallest ODN with acceptable activity.
- Backbone modifications: to confirm specific activity of novel structures, controls incorporating these modifications must be re-evaluated for each target sequence.

for a direct indication of ODN activity[11]. An alternative approach is to construct a fusion between the target gene and a reporter gene (such as luciferase), so that reporter-enzyme activity becomes a surrogate marker for expression of the RNA sequence of interest[28]. A target gene can often be introduced into relevant mammalian cells by transfection so that its sequence can be manipulated, giving the researcher a powerful tool to evaluate antisense specificity. Although inhibition of cell proliferation was commonly used as a measure of antisense activity in the past, its use to assay ODNs must include rigorous controls, because of the variety of nonspecific effects induced by these molecules[8,29].

In vivo *models*

The most satisfactory proof of concept for an antisense approach is efficacy of the ODN therapy in an animal model of the relevant disease. Many antisense ODNs are active in human tumor-xenograft models, where they have been administered systemically[30,31] or locally[32]. Antiviral compounds can be evaluated *in vivo* if suitable infectious models are available. Animal models must also be used to evaluate the pharmacokinetics and toxicity of an ODN, but it can be difficult to design satisfactory *in vivo* RNA-target controls. Compounds showing sequence-specific efficacy *in vivo*, with a good therapeutic ratio, may be considered for evaluation in a Phase I trial.

Compounds in clinical trials

Oligonucleotides from seven companies have been approved for human trials (Table I). Isis Pharmaceuticals and Lynx Therapeutics are in the forefront of antisense therapeutics with five and three Investigational New Drug (IND) approvals, respectively. The FDA has approved 15 INDs primarily relating to antiviral, anticancer and anti-inflammation drugs; however, in contrast to the wealth of data published on *in vivo* activity of ODNs, results of human trials are not yet widely reported.

Antivirals

The most advanced antisense therapeutic is ISIS 2922, currently in a Phase III trial for CMV-induced retinitis[33]. The compound, a 21-mer PS ODN targeting viral early gene expression, showed efficacy in a Phase I/II study of 17 AIDS patients who were refractory to ganciclovir and foscarnet. In this study, ISIS 2922 was injected directly into the eye in doses of up to 300 μg weekly, resulting in local drug concentrations as high as 8 μM; results of a Phase III study now in progress should be available near the end of 1996. As for many compounds in clinical trials, it is not clear if the activity of ISIS 2922 in man results from an antisense mechanism. Antisense trials of an AIDS ODN therapy have indicated that Hybridon's phosphorothioate, GEM 91 (Ref. 34), is promising. Hybridon has shown that GEM 91 is safe at doses from 0.1 to 2 mg kg^{-1} day^{-1} given intravenously or orally, and an escalating-dose Phase I/II trial is under way in the USA and France. The compound is administered by slow intravenous infusion to avoid the hemodynamic effects reported in primates after bolus administration of phosphorothioates[16,17].

Results for HPV, the only other virus targeted in a clinical trial, have been less promising than for HIV and CMV. Isis has recently discontinued development of ISIS 2105 for anogenital warts, reportedly because an effective drug concentration could not be maintained in the skin[35]. This phosphorothioate was injected locally after surgical removal of the wart, and targets the E2 gene, which is required for viral genome replication and transcriptional regulation.

Aronex Pharmaceuticals' AR 177 is an oligonucleotide in Phase I trials that, unusually, was developed as an aptamer with a non-antisense mechanism[14]. AR 177 is a predominantly phosphodiester molecule, which inhibits the HIV integrase protein directly rather than by blocking its production (Figure 4). The strong secondary structure induced by 'guanine quartets' in the AR 177 sequence makes it stable in cells, unlike other phosphodiester backbones[14]. The G-quartet motif, which has been exploited by Aronex for its protein binding affinity and its stability, has complicated other studies owing to similar non-antisense effects[36].

Leukemias and lymphomas

Leukemias caused by mutated or overexpressed genes have also been attractive targets, with three compounds in trials[37,38]. Antisense therapies for acute and chronic myelogenous leukemia or follicular lymphoma have targeted c-*MYB* (Lynx), *p53* (Lynx) and the *BCL-2* gene on the Philadelphia chromosome (Genta/Baxter)[21]. For leukemias, delivery of ODNs to malignant cells by *ex vivo* bone marrow treatment may reduce drug toxicity associated with systemic administration. Initial results from the Lynx trial of OL(1)p53 suggest that this 20-mer PS ODN is well-tolerated[37,39], though efficacy has not yet been reported.

Solid tumor and other indications

Isis and Ciba-Geigy have collaborated on ISIS 3521/CGP 64128, an antisense PS ODN targeting *PKC-α*, which inhibits

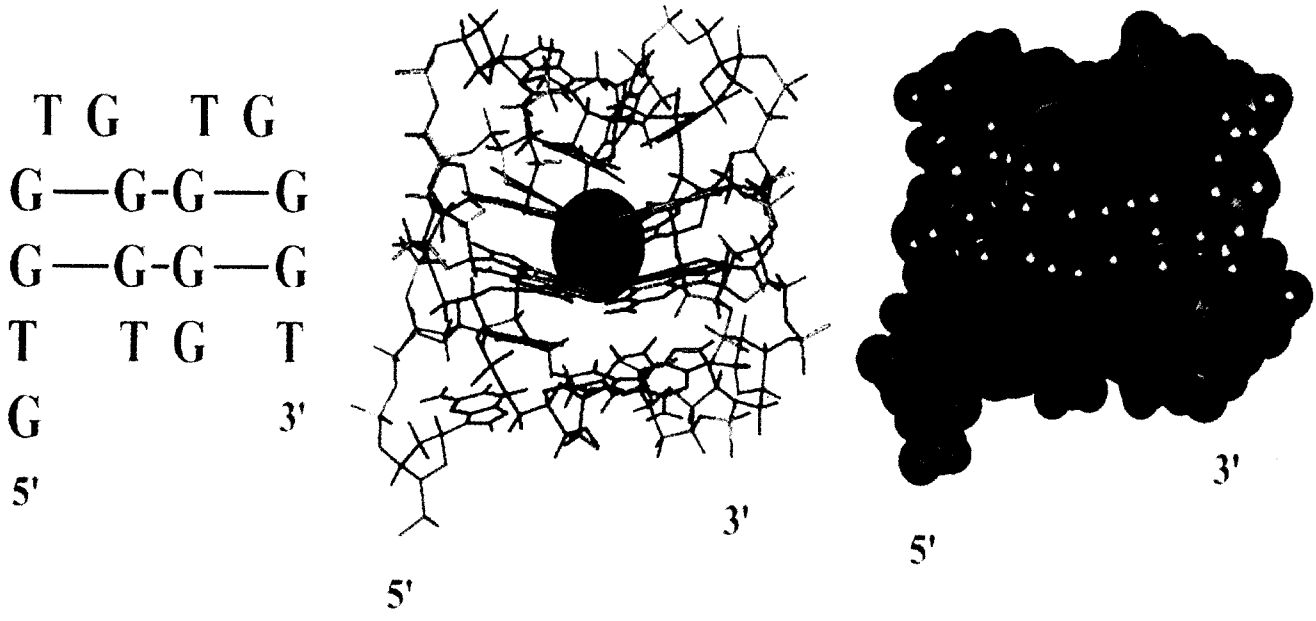

Figure 4. *Structure of an oligonucleotide with an aptamer (protein-binding) mechanism of action. The sequence d(GsTGGTGGGTGGGTGGGsT) (AR 177, an inhibitor of HIV integrase) is stabilized by two G-quartets. Quartet-forming guanosines are shown in gray, with other bases blue. The two stacked quartets are stabilized by a potassium cation (red). AR 177 is predominantly PO with one PS linkage at each end, but its stable secondary structure provides superior nuclease resistance relative to typical PO ODNs (reprinted with permission from Bishop* et al.*)*[14].

human tumor growth in nude-mouse models at doses as low as 0.006 mg kg^{-1}. Isis and Ciba-Geigy have also received IND approval recently for a phosphorothioate targeting the gene encoding for c-Raf kinase in solid tumor indications[31]. Because of the nature of solid tumor therapy, these drugs must be administered systemically. Isis is collaborating with Boehringer Ingelheim to develop an antisense molecule targeting the gene encoding ICAM-1, which is now in clinical trials for indications including rheumatoid arthritis, transplant rejection and inflammatory bowel disease[40]. Lynx's restenosis therapy 4003W94 targets c-*myc* to reduce smooth muscle cell proliferation, and can be delivered locally by catheter after balloon angioplasty[41].

The human pharmacokinetics, toxicity and efficacy results for these first clinical trials should be published in the next few years, and will largely determine the future of oligonucleotides as therapeutic agents. However, research on antisense gene therapy has already advanced well beyond these first phosphorothioates, and the modified backbones and drug delivery systems now in preclinical development hold the key to success for this technology.

Preclinical research and development

Conventional antisense strategies

A review of the extensive literature on oligonucleotide therapy indicates that many genes with relevance to disease have been targeted by antisense methods. Several conventional antisense ODNs are effective in animal models of human disease and could progress to IND approval in the near future (Table 2). Numerous gene targets implicated in cancers have been evaluated *in vivo*, including genes encoding for protein kinase A, NF-κB, p120, c-Myb, Ha-Ras, c-Raf kinase and BCR-ABL (Refs 2,4,31 and 38). Antiviral compounds in research or preclinical development include therapies for HSV (Ref. 42), hepatitis B virus (HBV) (Ref. 43), HCV (Ref. 44), Epstein–Barr virus (EBV) (Ref. 45), respiratory syncytial virus (RSV) and influenza virus[46]. Proof of an antisense mechanism is more difficult to assess in these animal models than in cell culture assays; however, in contrast to clinical trials, it is often possible to include appropriate controls to confirm sequence and target specificity. As an example, ODNs targeted against one isotype of protein kinase C or c-Raf kinase mRNA have no effect *in vivo* against expression of isotypes with different sequences[30,31]. With antiviral

Table 2. Selected companies with oligonucleotides in research and preclinical development

Companies	Targets	Strategies
Aronex Pharmaceuticals/ Hoechst Marion-Roussel	CMV, HSV, tumor necrosis factor, vascular endothelial growth factor	Aptamer, triplex, antisense
Atlantic Pharmaceuticals/ Gemini Gene Therapies	Respiratory syncytial virus, leukemias, dsDNA protein kinase	2-5A oligoadenylated antisense (RNase L activation)
Biopharmaceutic (USA)	HIV, solid tumors and metastases	Antisense
BioResearch Ireland	*MDR*-2 (multidrug resistance)	Antisense
Chugai Biopharmaceuticals	Tuberculosis, anticancer, antiviral	Antisense
CV Therapeutics	Restenosis (cell-cycle genes)	Antisense
Enzo Biochem	HBV	Antisense
Epoch Pharmaceuticals (MicroProbe)	Type I diabetes, autoimmune diseases (HLA genes), CMV, HIV (reverse transcriptase inhibitors)	Aptamer, gene-modifying ODNs (triplex, DNA-damaging)
Genset	HSV (*ICP*4 gene), HIV (*tat* and *tar* genes), NF-κB (inflammation), c-*myc* (anticancer)	Dumbbell transcription factor decoys, antisense
Genta/Baxter Healthcare Genta/Johnson & Johnson	HIV (*tat* and *tar* genes), HPV (E1, E2, E6, E7 genes), graft vs. host disease, *ERB*2, focal adhesion kinase, *BCL*-2 (anticancer), androgen receptor (acne, hair loss), interleukins (psoriasis), restenosis (after angioplasty)	Methylphosphonate antisense and triplex ODNs
Geron/Kyowa Hakko	Telomerase	Antisense
Gilead Sciences/ GlaxoWellcome	Thrombin (anticoagulant), anticancer, antivirals	Triplex and antisense
Hoechst Marion-Roussel	*BAX* mRNA (apoptosis)	Aptamer and antisense
Hybridon Hybridon/Searle (Monsanto) Hybridon/Medtronic	CMV, protein kinase A (solid tumor), VEGF (angiogenesis), influenza A, rheumatoid arthritis, amyloid precursor protein and β-amyloid protein (Alzheimer's), malaria	Hybrid and self-stabilized ODNs, oral formulations
ICN Pharmaceuticals	Psoriasis, anticancer, antiviral	Antisense
Immune Response/Chugai	HBV, Factor VIII gene	Antisense
Innovir	MDR, HIV, HBV, HPV, leukemias (APL and CML)	External Guide Sequences (RNase P activation)
Isis Pharmaceuticals Isis/Boehringer Ingelheim Isis/Ciba-Geigy Isis/Kaketsuken-Mochida	HIV, HCV, EBNA-1 (EBV in Burkitt's lymphoma, nasopharyngeal carcinoma), p120, PKC-α, c-*raf* kinase (anticancer), HPV (E6 and E7 genes), *MDR*-1/*MRP* genes, *VCAM*-1 (inflammation), Ha-*ras*, Ki-*ras* (anticancer)	Peptide–nucleic acid triplex, chimeric antisense, coupled RNA cleavage agents
Johnson & Johnson	HIV	Transcription factor decoy, antisense
Lynx Therapeutics Lynx Therapeutics/Chiron Lynx Therapeutics/NCI Lynx Therapeutics/NIH	HBV, HCV, HIV, restenosis, ovarian cancer, β-*FGF* in Kaposi's sarcoma	Phosphoramidate ODNs, triplex, antisense
MethylGene/Hybridon	DNA methyl transferase inhibitors (colon, breast cancer, other solid tumors)	Antisense
NeXstar	Angiogenesis, selectin, keratinocyte growth factor (psoriasis), inflammation	Aptamers (combinatorial ODN libraries), antisense
OncorPharm	β-adrenergic receptor (obesity), sickle cell anemia, cystic fibrosis, adenosine deaminase, anticancer, antiviral	Triplex, ODN-directed DNA damage/repair/recombination
Prizm	Kaposi's sarcoma	Mitotoxin-conjugated antisense
RGene Therapeutics	*BCR-ABL* (CML, other leukemias)	Antisense
Roche Roche/Hybridon	NF-κB, telomerase (anticancer) HBV, HCV, HPV	Antisense
Sankyo	Malignant melanoma, HIV	Dimethyoxytritylated ODNs
Sennes Drug Innovations	Cell proliferation	Antisense
SRC VB Vector	HIV	Alkyl- or lipid-conjugated ODNs
Texas Biotechnology	Restenosis (after angioplasty)	Antisense
Theratechnologies	HIV	Antisense
VimRx Pharmaceuticals/ Ribonetics	Amelogenin, *MDR*-1, leukemias	Catalytic antisense
Yissum	Cancers, thrombocytotic syndromes	Antisense

ODNs, activity against different strains of virus can be compared; for example, compounds targeting HSV-1 *in vivo* are less effective against HSV-2, which has an altered target site[42]. However, in several of these examples, including NF-κB (Refs 29,36) and c-*myb* (Ref. 38), ODNs active *in vivo* contain sequence motifs (such as G-quartets) that induce non-antisense effects, and these may contribute to biological efficacy. Based on other phosphorothioate molecules previously given IND approval, several of the above compounds will enter clinical trials assuming that their safety and efficacy are similar to current Phase I ODNs, regardless of *in vivo* mechanism of action.

Novel strategies

All compounds approved for clinical trials incorporate phosphodiester or phosphorothioate backbones, and most have been developed using conventional antisense approaches. By contrast, the latest second-generation compounds employ innovative alternatives to the use of unmodified ODNs as inhibitors of gene expression. A novel approach from Atlantic Pharmaceuticals involves the recruitment of a cellular ribonuclease to the selected RNA target by attachment of 2′,5′ oligoadenylate (2–5A) to an antisense ODN, creating a bifunctional molecule. The ODN binds the RNA of interest, while the conjugated 2–5A group induces target cleavage by RNase L, an interferon-inducible ribonuclease normally involved in degradation of single-stranded viral and cellular RNA (Ref. 47). Activity has been demonstrated against RSV and protein kinase R in cell assays. A similar approach by Innovir alters the normal substrate specificity of another ribonuclease by using 'External Guide Sequences' to induce degradation of target RNA (Ref. 48). RNase P is a ribonuclease responsible for tRNA processing; therefore, if a short sequence resembling its tRNA substrate is linked to an antisense ODN, its nuclease activity can be redirected to any mRNA.

Other intriguing modifications of antisense technology have been exploited by Epoch Pharmaceuticals and OncorPharm. Both groups link specific triple-helix-forming ODNs to a DNA-damaging agent, thereby inactivating or possibly stimulating repair of a mutated or overexpressed gene[49]. Another variation on the antisense theme, under investigation by companies including Genset and Johnson & Johnson, uses duplex oligonucleotides as promoter sequence 'decoys' to sequester transcription factors, thereby preventing expression of genes regulated by the designated promoter[12].

***In vivo* properties of oligonucleotides**

A fundamental assumption of antisense technology has been that the pharmacokinetic and toxicological properties of compound sequence and backbone are independent. This generalization is unusual in orthodox drug design, and has resulted in the rapid acceptance of the phosphorothioate backbone for most antisense research since an initial report of efficacy against HIV (Ref. 50). Though a useful working approximation, it is unrealistic (but understandable) to assume that toxicity and pharmacokinetic data for one phosphorothioate compound will be identical to those for PS ODNs of different sequences.

Pharmacodynamics

An oligonucleotide must maintain an active concentration in the appropriate cell to reduce target gene expression. At this concentration, the therapeutic ratio between efficacy and toxicity should be estimated based on *in vitro* and *in vivo* data. In spite of their specificity, ODNs may interact with nonspecific cellular components on the way to their ultimate RNA target. ODNs must first pass through the blood, where phosphorothioates bind to serum albumin[51], activate complement and inhibit coagulation[52]. Next, PS ODNs must penetrate cells, where they bind membranes either nonspecifically or via surface receptors[53]. Naked ODNs are thought to enter cells via endocytosis and may be processed through an endosomal/lysosomal pathway[53,54], but it is not understood how 'naked' antisense ODNs subsequently reach the cytoplasm. Once in the cytoplasm and nucleus, ODNs can interact with multiple components in addition to RNA, including transcription factors and immunoglobulins[29,36]. The phosphorothioate backbone generates more sequence-nonspecific effects than the phosphodiester backbone, so one goal has been to minimize PS content, thereby reducing toxicity while retaining compound stability[55]. In addition, particular base motifs, including G-quartets[14,29,36] and CG dinucleotides[56], can induce sequence-specific non-antisense effects, which may cause biological activities unrelated to inhibition of expression of the target gene[8].

Distribution of radiolabeled ODNs has been studied in mice, rats, rabbits, monkeys and humans. ODNs accumulate in most organs, with the highest concentrations in liver, kidney and spleen[57,58], although ODNs do not cross the blood–brain barrier[57,59]. Different routes of administration produce similar uptake and distribution characteristics[60]; ODNs distribute via a two-compartment model, with rapid clearance from plasma into tissue, followed by slower

elimination from the body. Phosphorothioate ODNs are cleared from plasma with a half-life of less than 1 h, and are then eliminated from body tissues with a half-life of 35–50 h (Refs 35 and 57). In most species, doses of a few milligrams per kilogram result in peak plasma levels in the low micromolar range[57,60]. Compounds with modified backbones generally distribute with similar pharmacokinetics, but can remain intact much longer than phosphorothioates[58,61]. Solid tumor indications may pose a greater challenge for antisense uptake than targets such as leukemia or HIV; however, Plenat and coworkers[62] have shown that antisense ODNs penetrate all cells of solid human tumors implanted in nude mice.

With advances in medicinal chemistry, antisense technology may have reached a point where compound stability is not a limiting factor for *in vivo* activity, because compounds may be cleared from the body faster than they are degraded[58]. The encouraging success of recent ODN structural modifications suggests that the current critical objective is to improve compound uptake and retention in the target cell.

Toxicity

Oligonucleotides have been administered to hundreds of patients in all phases of clinical trials (Table 1), with few adverse side effects[34,35,37]. Trials have administered ODNs at doses ranging from 150 μg per eye every two weeks (ISIS 2922 for CMV retinitis) to 6 mg kg^{-1} day^{-1} for 10 days (Lynx OL(1)p53 for acute myelogenous leukemia)[39]. However, regardless of their intrinsic specificity, ODNs induce definite class-related toxicities *in vivo* at doses disturbingly close to those administered in human trials[16,63].

It has been reported that ODNs are generally not toxic in cell cultures below 100 μM, and that the acute LD_{50} for phosphorothioates in mice is greater than 500 mg kg^{-1} for several sequences and routes of administration[2]. However, other groups have reported deaths in mice at doses as low as 100 mg kg^{-1}, and ODNs can be toxic in primates at substantially lower doses[3,16,17]. Generally, intraperitoneal dosing induces greater toxicity than slow intravenous or subcutaneous administration, possibly because of a localized inflammatory response. Significant species-specific differences exist for ODN toxicity. For example, in monkeys, but not lower mammals, hemodynamic changes (including severe hypotension) can occur at doses as low as 5 mg kg^{-1}, depending on the route of administration. Four separate IND applications to the FDA showed that monkeys die after bolus injection of relatively low doses of phosphorothioates[16,17,63]. These results from several independent studies understandably alarmed scientists in the antisense field (and the FDA); however, although these effects are still not completely understood, they can be eliminated by slower drug infusions.

Antisense compounds commonly induce splenomegaly in mice, possibly because of stimulation of the immune system (including B cell proliferation, interferon induction and increased natural killer [NK] cell activity) by particular sequence motifs[56,64]. Owing to the nature of oligodeoxynucleotides, genotoxicity has been a concern; however, compounds are neither mutagenic nor teratogenic in mice[65,66]. Because antisense compounds accumulate in the liver and kidney, where ODNs are degraded and eliminated, toxic effects are often first detected in these organs[57]. Death in mice and rats at high doses of phosphorothioates usually results from kidney failure[3]. If drug safety studies address the above toxicological issues, a lack of evidence for verifiable antisense activity *in vivo* does not necessarily preclude a successful IND application. In addition, any change in target sequence, length or backbone is considered a new molecular entity with regard to the IND application, so the exact lead sequence and backbone must be identified before final development decisions.

Medicinal chemistry

Medicinal chemistry plays an essential role in antisense development, as in any small-molecule drug program. However, antisense is unique in that ODN backbone structure can be altered independently of 'active' base sequence, to enhance the therapeutic ratio while retaining Watson–Crick specificity. An important bonus is that improvements to one compound may be extrapolated to the design of unrelated ODNs. To improve on the phosphorothioate, new chemical entities must be safer, more active, and suitable for patent protection. Antisense technology has significantly advanced because of the PS backbone: it is stable, effective and easy to synthesize. However, limitations due to toxicity and poor uptake suggest that improved second-generation backbones will supersede PS ODNs. Many companies, including Isis, Ciba-Geigy, Sandoz, Gilead, GlaxoWellcome, ICN, Hybridon and Roche, have developed novel oligonucleotide backbones (Table 2), and it is likely that some of these compounds will soon enter clinical trials.

Variations of the sugar, phosphate and base are three possible chemical approaches to improve an oligonucleotide.

One goal has been to increase uptake and reduce toxicity by substituting an uncharged methylphosphonate or phosphoramidate backbone for the phosphorothioate[2,67] (Figure 2b, 2d). Another goal has been to reduce degradation of PS ODNs. Although much more stable than the phosphodiester, the phosphorothioate is not completely resistant to nucleases[57,68]. Stability is increased for ODNs containing self-complementary regions that hybridize and therefore resist single-strand deoxyribonucleases[69]. The 2′ sugar position has been a useful target for structural modification; for example, 2′ *O*-alkyl modifications (Figure 2h–k) can increase ODN:RNA hybrid stability, while inhibiting nuclease degradation. Minor base alterations can increase hybrid stability and nuclease resistance or reduce immune effects, while preserving normal hydrogen bonding (Figure 2n–q). Several of these improvements can be incorporated into one ODN molecule to simultaneously improve several pharmacological characteristics. Hundreds of different oligonucleotide structures have been described in the literature, and most retain partial phosphorothioate character. However, in a few radical approaches the phosphate–sugar backbone has been eliminated completely, while retaining the four natural bases. An example is the peptide–nucleic acid (PNA) backbone (Figure 2r and cover), which binds DNA and RNA at least as well as a PO oligonucleotide at normal ionic strength[70]. PNA molecules have the potential to form triplexes with DNA and A-form duplexes with RNA[71], but are uncharged and do not stimulate RNase H degradation of target RNA.

Evaluating the role of RNase H activity in biological efficacy is essential when developing new backbones. ODNs catalyze this ribonuclease only when they contain both a charged phosphorus backbone and a deoxyribose sugar[10,72]. Therefore, modifications that eliminate this charge or alter the sugar (Figure 2) to improve drug safety or uptake may reduce overall biological efficacy. An approach to overcome this problem is to incorporate several modifications into different parts of the molecule, each with a unique contribution to activity. Such hybrid ODNs usually contain modifications at the 5′ and 3′ ends to block nuclease degradation and reduce charge, while retaining an RNase H-sensitive phosphorothioate deoxyribose core[55,58,73].

Antisense delivery

Inefficient uptake of oligonucleotides into the cytoplasm or nucleus has been a significant impediment to antisense therapy. ODNs are much larger than typical drugs, and the negative charge of the common PO and PS backbones

Table 3. Delivery prospects for oligonucleotides

Approach	Companies
Cationic lipids	Gilead, Genta, Isis, Aronex, others
Fusogenic cationic lipids	Gilead/GlaxoWellcome
Covalent lipophilic attachments (e.g. cholesterol) (HIV, cancer, rheumatoid arthritis)	SRC VB Vector, Zynaxis (Aronex, NeXstar), Genta, Isis
Neutral liposomes (HIV, CML, other leukemias, restenosis)	Epoch (MicroProbe), Aronex, RGene Therapeutics, Genset, NeXstar, CV Therapeutics, Yissum
Immunoliposomes	Isis/Ciba-Geigy
Cholesterol and polyethylene glycol conjugates (angiogenesis, inflammation, psoriasis)	NeXstar
Membrane fusogenic compounds (HBV, factor VIII)	INEX Pharmaceuticals
Carbohydrate cell permeation enhancers	Interneuron/Transcell Technologies
Cholera toxin-antisense conjugates	ImmunoVax
Liver targeting via asialoglycoprotein conjugates (HBV, Factor VIII gene)	Immune Response Corporation/Chugai Biopharmaceuticals, TargeTech
Implantable infusion of ODNs (Alzheimer's)	Medtronic/Hybridon
Biodegradable sustained-release polymers, cyclodextrin (HIV)	Hybridon
Viral pseudocapsids	RPMS Technology
Direct injection	Chugai, Isis, Hybridon, Lynx, others
Poly(L-lysine)	Genset

effectively eliminates cell-membrane penetration[22,74]. With recent advances in medicinal chemistry that greatly improve ODN stability, delivery has become the limiting factor in achieving the *in vivo* concentrations required for efficacy[54].

Therapeutic targets in clinical trials (Table 1) were chosen at least partly with delivery in mind; for example, routes of administration have included local injections for HPV, CMV and restenosis, *ex vivo* treatment of bone marrow for leukemias, and systemic intravenous delivery only when necessary (such as for AIDS). Oral bioavailability, though demonstrated in rats[75], remains an ambitious goal for human therapy. Clearly, if antisense technology is to fulfill its promise as a comprehensive approach to human disease, innovative new delivery technologies are required (Table 3).

Liposomes and cationic lipids

Liposome formulation has been a major focus of ODN delivery efforts[76], with compounds encapsulated in neutral, pH-sensitive and targeted liposomes[77]. Neutral liposomes have shown promise, with Aronex Pharmaceuticals' Phase I HIV compound delivered systemically in such a formulation. pH-sensitive liposomes can incorporate membrane-destabilizing peptides to lyse the endosome and release its contents into the cytoplasm before ODNs can be degraded[78]. Liposomes containing antibodies for specific cell-surface markers offer the potential to direct ODNs to target cells; for example, antibodies to MHC class I molecules have been used to target HIV-1 ODNs to T cells[79]. Unfortunately, these antibodies are foreign proteins that may stimulate an immune response against the coated liposomes; in addition, the high cost and small amount of ODNs delivered by immunoliposomes are barriers to development.

By far the most common and successful route to deliver anionic PO and PS ODNs in cell culture is through interaction with cationic lipids. Several lipid formulations are commercially available, and their use is now routine for *in vitro* studies[77]. Although lipids can deliver oligonucleotides *in vivo*[59], this is generally not required in animal models[30,62]. In fact, cationic lipids can be more toxic than the oligonucleotide they deliver.

Lipid delivery prevents ODNs from becoming trapped in endosomes by inducing direct uptake through the cell membrane into the cytoplasm, and ultimately into the nucleus[77,80]. A recent report from Gilead and GlaxoWellcome on a new cationic lipid/fusogenic agent that delivers ODNs to the nucleus with high efficiency shows that significant advances are still possible in this area[81].

Targeted delivery

ODNs can interact with specific cells through direct conjugation of the antisense compound to antibodies or to proteins for which surface receptors exist[82]. A two-phase strategy of targeting an antisense ODN to a specific gene in a specific cell should reduce overall toxicity. This approach has been used to target asialoorosomucoid-conjugated HBV-antisense to hepatocytes via their unique asialoglycoprotein receptors, so that ODNs are transported into the cell by receptor-mediated endocytosis[83]. Transferrin has been conjugated to ODNs to induce uptake into human leukemia cells via its receptor[84], and likewise folate conjugation has been used to internalize antisense to the epidermal growth factor gene via the folate receptor[85]. Lipophilic conjugates such as cholesterol can also improve uptake, stability or other properties[86].

Physical targeting is an alternative to these molecular methods. Microprojectile 'gene guns', for example, can deliver nucleic acids as vaccines[87] and could conceivably be used for ODN delivery. ODNs have also been adsorbed onto the surface of cyanoacrylate nanoparticles and other biodegradable polymers to protect them from intra- and extracellular nuclease degradation, and stimulate uptake via a phagocytic pathway[88].

Prospects for success of antisense technology

In the early 1990s, antisense oligonucleotides were promoted as 'magic bullets' that would make drug design as simple as reading a gene sequence. Although the first publications did not always demonstrate a convincing antisense mechanism, the field has progressed rapidly and sequence-specific efficacy is now a reality *in vitro* and *in vivo*. However, careful target choice and controlled studies are always required to demonstrate a genuine sequence-specific reduction in gene expression. The reported efficacy of ISIS 2922 in a Phase II trial for CMV infection has shown that antisense technology can be developed from first principles through to a drug with therapeutic value.

Oligonucleotides have been a greater challenge to develop than typical small-molecule drugs largely owing to their size and charge. To show efficacy *in vivo*, ODNs must be stable in plasma and in the cell, penetrate cell and nuclear membranes, and finally bind to the target RNA to block its expression or stimulate its degradation. ODNs that satisfy all these requirements must also possess a satisfactory therapeutic index and be economical to manufacture and practical to administer.

Economics of ODNs

Currently, the cost of oligonucleotide synthesis, over and above other considerations, may preclude acceptance of antisense technology. Antisense compounds are expensive, and their poor uptake implies that large doses will be needed. However, costs for ODNs have dropped dramatically in the past few years as companies have optimized syntheses of kilogram quantities for clinical trials[89]. A typical trial of a systemically-delivered oligonucleotide might require 10 kg of compound per year. Hybridon's goal, for example, is to produce (to GMP standards) 1,000 kg annually of its AIDS therapy GEM 91. Therefore, Hybridon and Pharmacia BioTech have collaborated to design new machines capable of 100 mmol syntheses, and report that they have produced 350 g of GEM 91 in 14 h with a yield of approximately 80%. Genta is also developing large-scale ODN production methods, and has reported that synthesis costs dropped from $35,000 per gram in 1988 to $250 per gram last year.

Corporate collaborations

Many larger pharmaceutical companies have external collaborations with biotechnology companies that develop innovative products but often lack the resources to support a compound in clinical trials. GlaxoWellcome/Gilead, Roche/Hybridon, Ciba-Geigy/Isis and Wyeth-Ayherst/Genta are just a few of these agreements from the last few years (see Tables 1, 2 and 3). Numerous patents for the concept of antisense technology[90], for specific medicinal chemistry modifications[91] and for particular gene targets[92,93] have been filed or granted for oligonucleotides, with important patents held by companies such as Gilead, Hybridon, MicroProbe (now Epoch Pharmaceuticals) and Isis. Key NIH patents relating to phosphorothioate ODNs have been licensed to Gilead, Lynx, Genta, Isis and Hybridon.

Regulatory issues

Oligonucleotides are classed as chemical, not biological, entities so clinical trial requests are evaluated by the FDA's Center for Drug Evaluation and Research (CDER), rather than Biologics (CBER). ODNs are judged using the same standards as other drugs, though they also share characteristics of vector-based and ribozyme gene therapy. Representatives of the FDA have provided excellent advice to sponsors of oligonucleotide INDs, both in publications and at several conferences. In general, the FDA does not require demonstration of an *in vivo* antisense mechanism prior to clinical trials; however, if such a mechanism is claimed, then supporting data must be provided. Regardless of mechanism, *in vivo* safety and efficacy are the primary requirements for clinical trial approval[63,94]. Proper toxicity studies must be included, focusing particularly on known or suspected risks intrinsic to PS ODNs, such as cardiovascular and mutagenic effects. Because nonspecific toxicity has become apparent, particularly with the deaths of primates treated with PS ODNs, additional monitoring is now essential[63]. Genotoxicity will remain an issue, especially as the new backbone and base modifications may be more stable and mutagenic than phosphorothioates. Finally, pharmacokinetic and toxicological parameters must be evaluated using the formulation and route of administration intended for clinical trials. The FDA is well aware that PS ODNs may cause serious toxic effects *in vivo*[63,94], and the pharmaceutical industry must address their concerns.

The ultimate goal of antisense oligonucleotide therapy is to rationally generate effective new drugs, while reducing development time and cost relative to orthodox drug discovery programs. A critical advantage of ODN therapy is that the techniques developed on one project can be applied to new projects, whether in oncology, infectious agents, inflammation or any disease where a gene is foreign or overexpressed. To generate novel and active compounds, antisense technology fundamentally requires only a target-gene sequence and the knowledge that A pairs with T and G pairs with C, which makes this approach an attractive complement to traditional drug discovery methods.

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