Emerging antisense technologies for gene functionalization and drug discovery

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In February 2001, the efforts of the Human Genome Project (HGP) resulted in the publication of a 'working draft' of the entire human genome. It is expected that final sequencing and annotation of the genome will be completed within two years and the HGP is beginning to focus on identifying the functions of thousands of novel genes. During the past few years, antisense oligomers have been widely used as potent tools for functional genomics and drug target validation. This article describes the established and emerging antisense technologies that will continue the efforts to unlock the function of the human genome.

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▼ The human genome project (HGP) has been described as the single most important project in biology and biomedical sciences to date[1]. Since its inception in 1990, the HGP has revolutionized the ways in which biologists think about the organization of the human genome and the methods used to identify the functions of novel genes. Using bioinformatics, it is now possible to ascertain the probable function of a gene without performing a single experiment. The HGP has had a profound impact on the drug-discovery efforts of biotechnology and pharmaceutical companies. During the past decade, the HGP has facilitated advances in microarray technologies that enable thousands of genes to be profiled in a single experiment, and which are widely used today to identify interesting genetic and biochemical pathways. With the completion of the sequencing, numerous novel potential drug targets will be identified and evaluated in cell culture and in animal models of human disease. The competition between companies to be the first to identify and validate potential drug targets is tremendous, and technologies that enable efficient mining of the sequence data from the vast repositories in the public and private databases are in high demand. Inhibition of a target gene is the most direct approach to determining its function. A number of technologies that enable knockdown or knockout of gene targets are currently in use, including saturation mutagenesis[2,3], targeted-gene knockouts[4], small-molecule inhibitors[5] and antisense oligonucleotides.

Antisense technology is well suited for gene functionalization and drug target validation because it is specific and broadly applicable. Furthermore, antisense compounds that are effective inhibitors of gene function can be designed and synthesized with minimal sequence information (e.g. expressed sequence tags). The distinct advantages that antisense technology offers compared with competing technologies are that (1) antisense effects are rapidly detected; (2) the role of a gene in the adult animal can be determined in cases in which the use of knockout technology results in embryonic lethality; (3) phenotypic changes caused by inhibition of target genes can be readily evaluated in cell culture models of disease; and (4) the specificity of antisense oligonucleotides allows for discrimination among closely related homologs[6–10].

Antisense technologies

Antisense oligonucleotides are short (7–30 nucleotides) sequences of nucleic acid designed to bind to a specific region of a target mRNA. Oligonucleotides bind to the target mRNA according to Watson–Crick base-pairing rules, enabling rational design of antisense compounds to inhibit any gene target, provided that the sequence is

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known. The mechanism of action by which antisense oligonucleotides inhibit the translation of mRNA depends on the oligonucleotide chemistry (Fig. 1). For example, oligonucleotides consisting of regions of DNA (e.g. phosphodiester, phosphorothioate and chimeric) can act by recruiting endogenous RNase H, an enzyme that recognizes RNA–DNA duplexes and cleaves the RNA strand. Modified antisense oligomers that do not recruit RNase H (e.g. peptide nucleic acids, morpholino) can inhibit protein synthesis by hybridizing in the 5′ UTR or near the AUG start codon, which blocks ribosome assembly[11,12]; such oligomers can also inhibit splicing when hybridized to intron–exon junctions[13].

In 1978, Zamecnik and Stephenson[14,15] presented the first experiments describing the use of antisense oligonucleotides to inhibit a target gene. The technology was immediately touted as a panacea for gene-function analysis and for the development of therapeutics. Many of the early results using first-generation oligonucleotides were irreproducible, and called into question the broad applicability of the technology. The failings of the early antisense experiments were caused by the use of unreliable transfection conditions and sub-optimal antisense chemistries. For example, the use of phosphodiester oligonucleotides was largely unsuccessful because of their susceptibility to degradation by endo- and exonucleases. Replacement of phosphodiester oligonucleotides by phosphorothioate oligonucleotides solved the issue of stability, but did not completely eliminate the irreproducible antisense results. In many cases, investigators applied high concentrations of phosphorothioate oligonucleotides to cells, leading to nonspecific activity of phosphorothioate oligonucleotides, including phenotypic changes caused by interaction with cellular proteins and nonspecific cleavage of unintended targets[16]. Phosphorothioate oligonucleotides are still widely used today; however, the use of chimeric oligonucleotides is gaining favor because of their improved properties.

Chimeric oligonucleotides contain a combination of deoxynucleotides and modified oligodeoxynucleotides, oligoribonucleotides and/or modified internucleotide linkages[17–19]. These oligonucleotides are designed to take advantage of both the RNase H activation of the deoxynucleotide ‘gap’ and of the improved binding affinity of the modified DNA or RNA stretches[18–20]. Chimeric oligonucleotides exhibit enhanced binding and antisense activity[6,17–19,21] and reduced toxicity[17] compared with phosphorothioate oligonucleotides.

In recent years, a consensus has emerged in the field about the importance of performing carefully controlled experiments to avoid confounding results. The mechanisms of action of antisense oligonucleotides must be demonstrated by direct inhibition of the target gene at the RNA or protein level by several antisense oligonucleotides directed against different sites on the target RNA, and by lack of inhibition by several scrambled and mismatch control oligonucleotides. Advances in antisense oligonucleotide chemistry and in methodologies for the delivery of oligonucleotides to cells have led to reproducible results showing that antisense oligonucleotides cause potent and specific inhibition of target-gene expression[6–8,10,22,23]. Based on a large body of literature demonstrating its efficacy and broad applicability, antisense technology is now considered a proven technology for elucidating target-gene function and for the identification of novel drug targets.

Antisense for functional genomics and drug target validation

One application for antisense technology is to validate drug targets. Although the phenotypes of many diseases are well known, identification of the genes responsible for those phenotypes remains a major hurdle in the drug development process. Historically, drug targets were identified and validated by screening large numbers of small-molecules designed to inhibit the function of particular genes. The development of small-molecule inhibitors requires substantially more information than is required to design antisense oligonucleotides. Many small-molecules interact with multiple members of a gene family, confounding the validity of the intended gene as a drug target. Antisense technology enables researchers to elucidate the roles of gene products within cellular pathways by specific inhibition of individual or simultaneous inhibition of multiple members of a gene family in their experimental system.

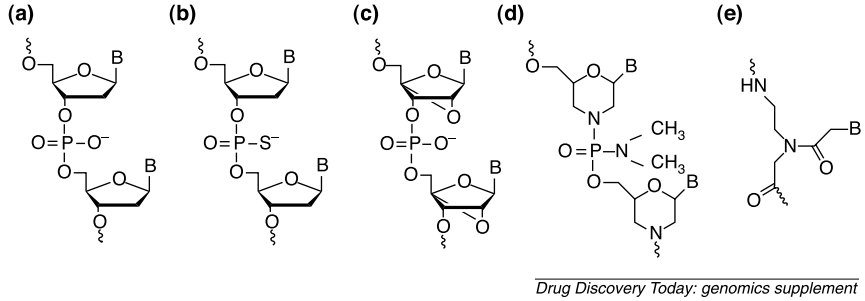

Figure 1. Illustration of selected oligonucleotide chemistries. (a) Unmodified DNA, (b) phosphorothioate DNA and (c) locked nucleic acid antisense oligonucleotides are reported to inhibit protein production via recruitment of RNAse H. (d) Morpholino and (e) peptide nucleic acid oligomers are reported to inhibit translation by blocking ribosomal assembly by hybridizing near the AUG start site or by interfering with splicing by hybridizing at intron–exon junctions.

During the past few years, antisense technology has played a key role in drug discovery and target validation efforts based on sequence information gathered from the HGP (Refs 6–8, 10,22,23). Many pharmaceutical companies are employing the technology to identify the function of novel sequences mined from the private and public databases. The versatility of the antisense approach makes it attractive for use in drug discovery efforts. Antisense oligonucleotides, in conjunction with the appropriate phenotypic assay, provide a rapid method for screening potential drug targets for various diseases (Fig. 2).

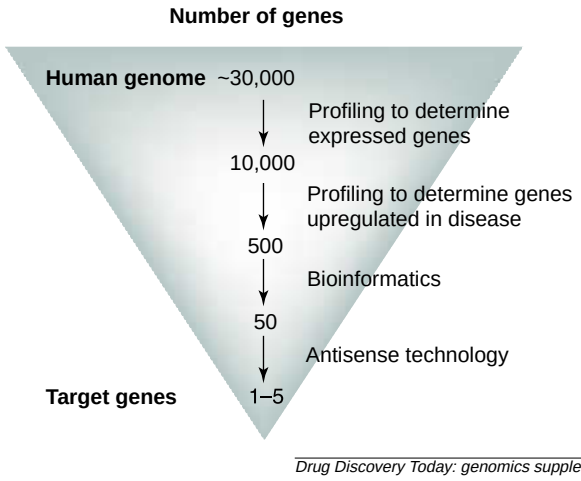

Figure 2. Diagramatic representation of the role of antisense technology in drug discovery. Antisense technology can be used to identify rapidly the most promising drugable targets that are identified by transcriptional profiling and differential expression of the reported 30,000 human genes.

Alzheimer's disease

In 1999, Yan *et al.*[22] and Vassar *et al.*[23] independently used antisense technology to implicate β-secretase as a novel and potentially important drug target for the treatment of Alzheimer's disease. In carefully controlled experiments, it was shown by both groups that inhibition of the β-secretase target by several antisense oligonucleotides that targeted different sites within the β-secretase mRNA, resulted in a decrease in amyloid-β peptide processing. No effects were observed with the use of reverse sequence or mismatch control oligonucleotides[22,23]. Recently, it was demonstrated that mice deficient in the Alzheimer's β-secretase (BACE) have normal phenotype and abolished β-amyloid generation, confirming BACE as a valid drug target[24].

Signal-transduction pathways

Antisense oligonucleotides have also been widely used in the elucidation of the biochemical pathways involved in cellular processes such as cell signaling, apoptosis, and cell-cycle regulation. Many diseases are the result of defects in the cell signaling pathways. A detailed understanding of the complex biochemical pathways governing signal transduction is key to the development of effective therapeutics for the treatment of human disease. Many, if not all, signal-transduction pathways involve the interaction of highly homologous genes. A limiting factor in the identification of gene function in signal-transduction pathways has been the ability to inhibit one isoform of a gene selectively while leaving the function of other isoforms intact. Antisense is ideally suited for and has been successfully used to elucidate the function of genes in various signal-transduction pathways (reviewed in Ref. 7). Recently, antisense technology has been applied to determine the unique functions of the jun N-terminal kinase (JNK) family of genes. The JNK pathway has been implicated in regulating the growth of tumor cells. Using antisense oligonucleotides that specifically inhibited either *JNK1* or *JNK2* expression, Potapova, *et al.*[7] demonstrated that both kinases, but particularly JNK2, are important regulators of the growth of human tumor cells that are deficient in *p53*. In a separate study[25], oligonucleotides were used to simultaneously inhibit JNK1 and JNK2 function. Complete inhibition of the JNK pathway abrogated epidermal growth factor-stimulated growth of A549 cells, again suggesting a growth-promoting role for the JNK pathway in human cancer cells.

The cell signaling pathways involved in apoptosis have also been intensely investigated because of their roles in many diseases, including cancer. The Bcl-2 family of proteins, consisting of members that can promote cell survival or cell death, has been of particular interest. Antisense oligonucleotides that effectively inhibit Bcl-2 in small-cell lung cancer cells effectively reduce cell viability and promote apoptosis[26]. These examples of gene inhibition using antisense oligonucleotides in carefully controlled experiments demonstrate the utility of the technology for target validation in cell culture-based models of disease states.

Determination of gene function *in vivo*

Once a potential drug target has been identified in a cell culture screen, the next step is to confirm the validity of the target through *in vivo* evaluation. The gold standard for determining gene function *in vivo* has been the use of genetic-knockout technology. Although genetic knockouts can be used to define clearly the role of a gene within the whole animal, the technology is not without limitations. The major limitations of knockout technologies for *in vivo* target validation are the length of time required to create and characterize the mutant strain, and the possibility of generating a lethal embryonic mutation, which precludes the evaluation of certain genes.

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Table 1. Antisense compounds currently in clinical trials[a]

Product	Company	Target	Indication	Stage of trial
Vitravene	Isis (Carlsbad, CA, USA)	Anti-viral	CMV-retinitis	On market
ISIS3521	Isis	PKC-α	Cancer	Phase III
Genasense	Genta (Berkeley Heights, NJ, USA)	Bcl-2	Cancer	Phase III
ISIS2302	Isis	ICAM-1	Crohn's disease	End of Phase II
ISIS2302	Isis	ICAM-1	Topical psoriasis	Phase II
ISIS14803	Isis	Anti-viral	Hepatitis C	Phase II
ISIS2503	Isis	H-ras	Cancer	Phase II
ISIS5132	Isis	Raf	Cancer	Phase II
Resten	AVI BioPharma (Portland, OR, USA)	c-Myc	Restenosis	Phase II
Oncomyc	AVI BioPharma		Cancer	Phase II
GEM231	Hybridon (Cambridge, MA, USA)	PKA (I)	Cancer	Phase II
ISIS104838	Isis	TNF-α	Rheumatoid arthritis, crohn's disease	Phase I
GEM92	Hybridon	GAG	HIV	Phase I
MG98	Hybridon	DNMT	Cancer	Phase I
Neubiotics	AVI BioPharma	Bacterial ribosomes	n.a.	Pre-clinical/ Phase I

[a]Abbreviations: CMV, cytomegalovirus; DNMT, DNA methyltransferase; HIV, human immunodeficiency virus; ICAM, intercellular adhesion molecules; n.a., not available; PKA, protein kinase A; PKC, protein kinase C; TNF, tumor necrosis factor.

Antisense oligonucleotides are an alternative method for identification of gene function *in vivo*. The effects of inhibition of target genes on the function of the whole animal can be determined rapidly. Also, the function of genes at various points in development, ranging from embryonic stages to adult animals, can be evaluated using antisense technologies[27–29]. In the past few years, antisense technologies have been used successfully for the validation of multiple targets in *in vivo* models. For example, interleukin(IL)-5 is a cytokine that has been implicated in the migration of eosinophils in the asthmatic lung. Using antisense oligonucleotides that target IL-5 in a well-characterized murine model of asthma, Karras *et al.*[30]. demonstrated that a reduction in IL-5 protein corresponded with decreased eosinophilia and improved airway function[30].

The proto-oncogene *c-Myc* is known to play a crucial role in the regulation of cell proliferation, differentiation and apoptosis. However, the role of *c-Myc* in the process of tissue regeneration is not clearly understood. Using a liver-regeneration model after partial hepatectomy, Arora *et al.*[31] showed that inhibition of Myc by antisense oligomers caused a dose-dependent and sequence-specific reduction of liver regeneration. In another study focusing on the effects in the liver, Zhang *et al.*[32] showed that antisense oligonucleotides targeting Fas offered a protective effect in a murine model of hepatitis.

It should not be overlooked that antisense oligonucleotides used for *in vivo* drug target validation could be further developed as therapeutic agents. Several antisense compounds are currently in clinical trials for the treatment of a broad spectrum of diseases, ranging from viral infection to cancer[10] (Table 1). Whereas antisense-based therapeutics might be practical for target genes expressed in tissues in which oligonucleotides accumulate at high levels (e.g. liver), efficient delivery of the oligonucleotides to other target tissues could limit the therapeutic potential of antisense technologies.

Conclusions

More than two decades after synthetic antisense oligonucleotides were first used to inhibit gene function, the technology is realizing its promise of a broadly applicable tool for gene functionalization. Antisense technology provides a rapid and specific method for determination of gene function, both *in vitro* and *in vivo*. The antisense approach could reduce the cost of drug discovery by expediting the identification of lead

targets for pharmaceutical intervention. Many of the limitations of first-generation antisense oligomers[33] have been overcome through advances in the technology leading to improved specificity and enhanced the stability of oligonucleotides. Transfection techniques have also been improved that enable delivery of antisense oligonucleotides to cells more reliably and with less toxicity than was previously possible. Antisense technology is now proven as a viable option for systematic and high-throughput determination of gene function and drug target validation.

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