# Antisense oligonucleotides: a systematic high-throughput approach to target validation and gene function determination

Margaret F. Taylor, Kristin Wiederholt and Fran Sverdrup

Antisense technology provides a high-throughput and systematic approach to drug target validation and gene function discovery. In combination with other emerging technologies (such as microarrays), this technology will enable efficient evaluation of the sequence data generated by the Human Genome Project. The authors review recent advances in the antisense field and discuss the potential use of antisense technology for functional genomics.

Antisense technology provides an elegant and simple approach to inhibiting the expression of a target gene. Antisense oligonucleotides (ONs) are short sequences (7–30 nucleotides) of nucleic acids that bind to a specific region of a target messenger RNA (mRNA), according to Watson–Crick base-pairing rules (Fig. 1) and can be designed to inhibit any gene target provided that the sequence is known. The specificity and ease of design of ONs make them attractive candidates as therapeutic agents and as research tools for the elucidation of gene function.

## First-generation oligonucleotides

Initial studies with first-generation ONs (mostly phosphorothioate-modified ONs) demonstrated that antisense ONs could inhibit gene expression in a sequence-specific manner. Although phosphorothioate ONs are more resistant to nuclease degradation than unmodified phosphodiester ONs (Ref. 1), phosphorothioate ONs are also associated with non-specific effects caused by interactions with intracellular and cell-surface proteins, non-specific cleavage of unintended targets[2], and transfection-induced toxicity. In addition, first-generation ONs were associated with some sequence-specific, but not antisense-mediated, phenotype modulation. These sequence-specific side-effects are termed aptamer effects. Two of the best characterized aptamer sequences are the G-quartet[3] and the CpG (cytosine followed by guanosine linked by a phosphodiester bond)[4] motifs. Non-specific and aptamer effects of first-generation antisense ONs have caused some to challenge their use as research tools for the elucidation of gene function[5].

Despite the controversy, phosphorothioate ONs are currently the most widely used and best characterized antisense agents. In the past two or three years, several phosphorothioate compounds have been evaluated in clinical trials for the treatment of diseases including cancer[6], Crohn's disease and rheumatoid arthritis, with promising results[7]. In 1998, the phosphorothioate ON, Vitravene (ISIS Pharmaceuticals, Carlsbad, CA, USA) became the first Food and Drug Administration (FDA)-approved antisense drug, currently used for the treatment of cytomegalovirus infection[8,9]. Several other antisense compounds with indications for the treatment of cancer, viral infection and inflammatory diseases are currently being evaluated in clinical trials (Table 1).

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 1359-6446/99/$ – see front matter  PII: S1359-6446(99)01392-6 

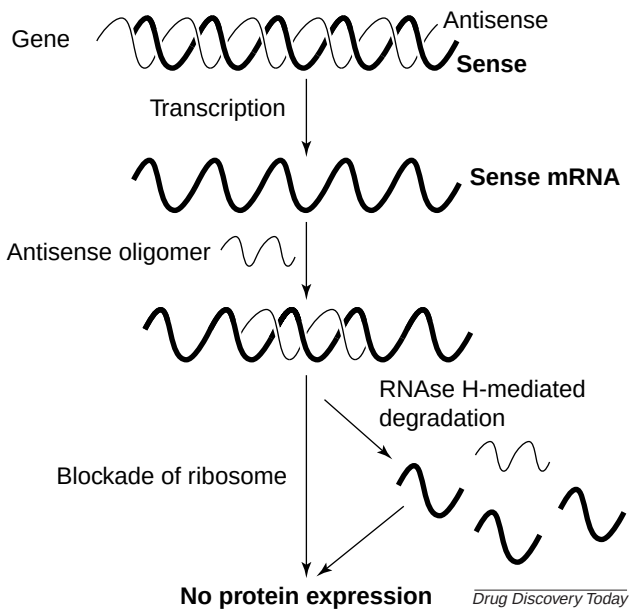


***Figure 1.*** *Schematic representation of antisense activity. The antisense oligomer binds to the target mRNA sequence according to Watson–Crick base-pairing rules, thereby inhibiting the translation of the target mRNA. The mechanisms by which antisense inhibits translation are either by physical blockade of the ribosome or by ribonuclease H (RNAse H)-mediated degradation of the target mRNA.*

In addition to advances in clinical applications, there has been a resurgence of interest in antisense technology for use in gene function discovery and for drug target validation. Advances in medicinal chemistry have led to the development of second-generation antisense ONs with increased specificity and lower toxicity compared with phosphorothioate ONs (Refs 10–12). Second-generation antisense technology is the foundation of functional genomics programs in several biotechnology and pharmaceutical companies. In the coming years, antisense technology is likely to have a key role in deciphering the genetic sequence information from the Human Genome Project, determining the function(s) of novel genes and validating potential drug targets.

**Table 1. Antisense oligonucleotides currently being evaluated in clinical trials**

| Company | Product | Status | Application |
|---|---|---|---|
| Hybridon | GEM92 | Phase I | Acquired immune deficiency syndrome |
| ISIS Pharmaceuticals | ISIS2503 | Phase I | Neoplasm, solid tumor |
| INEX Pharmaceuticals Corp. | INX3001 | Phase I | Leukemia |
| Genta | G1128 | Phase II | Neoplasm, leukemia |
| Genta | G3139 | Phase II | Cancer |
| Hybridon | GEM132 | Phase II | Cytomegalovirus infection, retinitis |
| ISIS Pharmaceuticals | ISIS2302 | Phase II | Multiple anti-inflammatory indications |
| ISIS Pharmaceuticals | ISIS3521 | Phase II | Neoplasm, solid tumor |
| ISIS Pharmaceuticals | ISIS5132 | Phase II | Multiple tumor targets |
| ISIS Pharmaceuticals | Fomivirsen | Approved | Cytomegalovirus infection |

## Second-generation antisense oligomers

Progress in nucleic acid chemistry has led to the development of modifications to improve the binding affinity and increase the nuclease resistance of phosphorothioate and phosphodiester ONs. The two most frequently described modifications are alterations of the internucleoside linkages (e.g. peptide nucleic acids and methylphosphonate) and of the 2′-O position of the ribose moiety. The incorporation of alkyl groups at the 2′-O position of ribose increases the binding affinity of ONs for their target RNA (Refs 13–17). A limitation of 2′-O-alkyl modifications is that ONs in which all of the 2′-deoxyribose positions are modified form RNA–ON complexes that are not substrates for ribonuclease H (RNAse H).

RNAse H is a ubiquitous cellular enzyme that recognizes RNA/DNA duplexes and cleaves the RNA strand. RNAse H-mediated degradation of the target mRNA is the most documented and best characterized mechanism of antisense action[18]. Oligomers that do not cleave the target by RNAse H can inhibit mRNA expression by interrupting splicing or by interfering with the translational machinery[19]. The potentially active target sites for inhibition by ONs that do not activate RNAse H are limited to the 5′-untranslated region, the AUG start codon and splice sites within the pre-mRNA (Ref. 20).

Second-generation ONs that retain the ability to activate RNAse include ONs with modified base residues and chimeric ONs. Incorporation of base modifications on cytosine, uracil[21–23] and adenosine[24] residues has been shown to enhance both the binding affinity and the activity of antisense ONs. Chimeric ONs (Refs 12,13,16) contain a combination of deoxynucleotides and modified oligodeoxynucleotides, oligoribonucleotides or

internucleotide linkages[12]. These ONs are designed to take advantage of both the RNAse H activation of the deoxynucleotide 'gap' and the improved binding affinity of the modified DNA or RNA stretches[11,13,16]. Chimeric ONs exhibit enhanced binding and antisense activity[12–14,16,25] and reduced toxicity[12] compared with phosphorothioate ONs. Several antisense companies are currently pursuing different chimeric oligomers as their lead compounds.

## Functional genomics

The Human Genome-sequencing Project has resulted in the deposition of a partial sequence of tens of thousands of genes in various expressed sequence tag (EST) databases. Methods for the rapid identification of gene function and validation of potential drug targets are required to efficiently utilize these databases. Functional genomics is a growing field in biotechnology, being used to determine the function of all genes and to elucidate the pathways governing the interaction and regulation of genes[26]. This field has attracted significant attention from the pharmaceutical industry as it is anticipated that the number of available drug targets will increase dramatically over the next few years[27].

Current approaches to functional genomics include comparison of sequence information with sequence databases for other organisms, saturation mutagenesis and targeted gene knockouts[26]. The creation of genetic knockout animals has been the 'gold standard' for the determination of gene function and knockout mice have yielded important information concerning the *in vivo* function of many genes. The major drawback of using knockout animals is the length of time taken from the generation of the embryonic stem cells carrying the mutation to the analysis of the animal. Another disadvantage is the potential for the generation of lethal embryonic mutations that preclude the evaluation of certain genes. As an approach to functional genomics, antisense technology offers several advantages over knockout technology:

- Antisense effects are rapidly detected
- The role of a gene in the adult animal can be determined (bypassing the embryonic lethal stage)
- Phenotypic changes caused by inhibition of human genes can be evaluated in cell culture.

## Antisense for functional genomics and drug target validation

Determination of the function of novel genes identified by the Human Genome-sequencing Project will be key in future drug discovery and drug development efforts, the most direct approach being by inhibition of a target gene. Efficient evaluation of the sequence data from the project will require a technique for gene inhibition that is specific, broadly applicable and can be designed with minimal information. Antisense technology meets these criteria. ONs can be designed to determine the function of novel genes based on minimal sequence information (EST sequence data are sufficient). Furthermore, the exquisite specificity of antisense technology enables the inhibition of one gene family member without affecting closely related members. Investigators from Isis Pharmaceuticals demonstrated antisense-mediated inhibition of the protein kinase C (PKC) which was selective for the α-isotype[13]. In a study conducted at Gilead Sciences (Foster City, CA, USA), Wagner and coworkers demonstrated the use of C-5 propyne antisense ONs to specifically inhibit the expression of cyclin B1-mRNA without affecting the expression of cyclin B2-mRNA (Ref. 28).

Antisense technology provides a systematic and high-throughput approach to the determination of gene function (Fig. 2). Step one of the antisense approach, which is selection and validation of target sites on the mRNA, can be completed very rapidly. The best target sites are still determined empirically, although improvements in the potency of ONs and in the algorithms used for predicting accessible

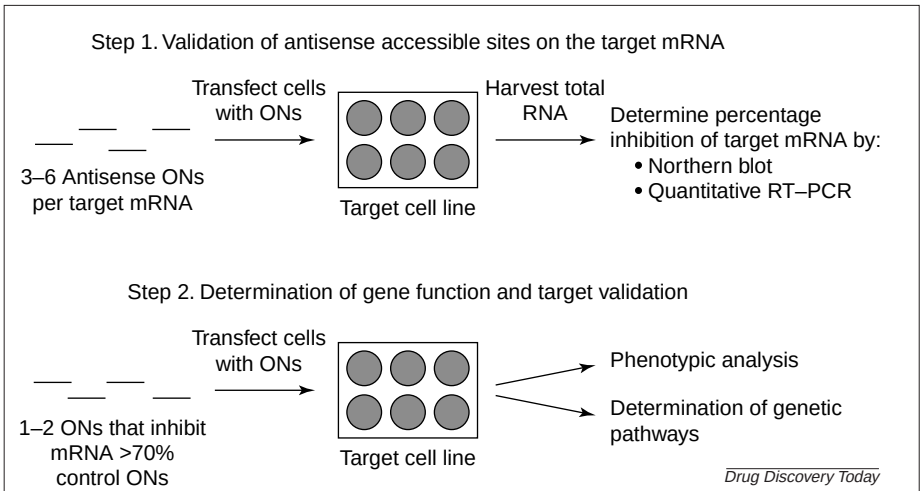


***Figure 2.*** *Antisense as a systematic high-throughput approach for gene function determination and drug target validation. Abbreviations: ON, oligonucleotides; RT–PCR, reverse transcriptase–polymerase chain reaction.*

sites on the target mRNA have drastically reduced the number of oligonucleotides that must be screened to find one that is effective. Previous recommendations required the screening of 30–60 ONs per gene. Using high affinity chimeric oligomers and a bioinformatics program to select accessible sites, Woolf and coworkers have found that screening 3–6 oligomers per target is sufficient to find one that inhibits the gene with 66–95% efficiency (Sequitur, Natick, MA, USA) (unpublished data), significantly reducing the time and labor required to identify active ONs.

To validate the target sites, ONs are transfected into cells. Most cell lines will accumulate antisense ONs in the nucleus after transfection by cationic lipid[29], microinjection[30] or electroporation[22]. Total RNA is harvested from antisense and control ON-treated cells, and the levels of the mRNA of interest are determined and compared with those of an internal control mRNA. Northern blot analysis has been the conventional method for RNA analysis, although advances in quantitative reverse transcriptase– polymerase chain reaction (RT–PCR) technology[31] allow high-throughput analysis of target mRNA inhibition to identify the most active ON(s). After showing inhibition at the mRNA level, phenotypic analysis or western blots can be employed to confirm the validity of the target site.

Examples of target mRNA inhibition caused by treatment with Version D antisense ONs (Sequitur) are shown in Figs 3 and 4 (unpublished data). The data in Fig. 3 show inhibition of β-actin mRNA by three specifically designed antisense ONs. Human epithelial-like (A549) cells were transfected with these ONs, and total RNA was harvested after 24 hours. Quantitation of a northern blot by densitometric analysis (Fig. 3) shows that ONs S10279, S10280 and S10281 cause significant reduction of the β-actin mRNA levels compared with the control ON (S10532). The Version D antisense ONs inhibit mRNA expression through RNAse H-mediated degradation of the target mRNA. This experiment demonstrates that antisense technology is useful even for the inhibition of extremely abundant mRNA targets. Oligomers S10280 and S10532 were further evaluated using a concentration-response study, which demonstrated that S10280 inhibits the expression of β-actin mRNA in a concentration-dependent manner (Fig. 4).

Following the identification of the most active ONs for a specific gene, they can be used to assay for phenotypic changes caused by loss of gene function. The time-course for phenotype evaluation is dependent upon the half-life of the protein of interest and could require extended periods of inhibition of the target mRNA (Ref. 32). Through a collaboration between Sequitur and Genome Therapeutics (Waltham, MA, USA), inhibition of target mRNA has been obtained for up to six days using proprietary transfection techniques (Mark Osborne, Genome Therapeutics, pers. commun.).

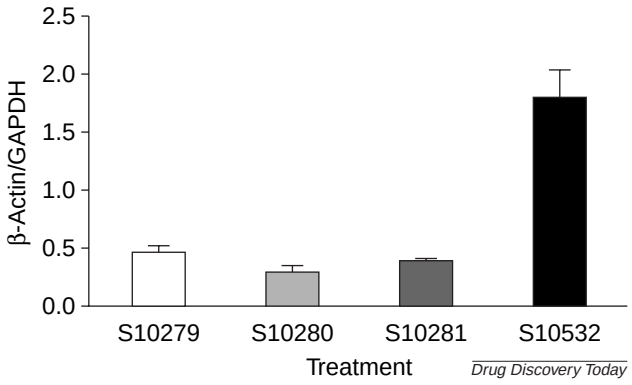


***Figure 3.*** *Inhibition of β-actin mRNA by the Version D chemistry (Sequitur, Natick, MA, USA) antisense oligonucleotides (ONs), S10279, S10280 and S10281, which were designed to act against β-actin. The human epithelial-like A549 cells were transfected with 40 nM of either antisense ONs or a non-targeted sequence control ON (S10532). Transfection was facilitated by Oligofectin G (2.5 μg ml$^{-1}$, Sequitur) in the presence of serum for 24 h. After transfection, total RNA was isolated from the cells and β-actin and glyceraldehyde phosphate dehydrogenase (GAPDH, internal control) mRNA levels determined by northern blot analysis and quantitated using Scion Image beta 3 (Scion Corp., Frederick, MD, USA). The expression of β-actin mRNA was normalized to the expression of GAPDH mRNA. Antisense sequences (S10279, S10280 and S10281) caused inhibition (75%, 85% and 78%, respectively) of the target mRNA compared with control ON (S10532). Data shown represent the mean ± SD of duplicate transfections.*

Antisense technology can also be used 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. Typically, drugs are developed by screening a large number of small molecules designed to inhibit the function of a particular gene. Rational design of small-molecule therapeutics requires substantially more information than is required to design antisense ONs. The exquisite specificity of antisense technology enables the evaluation of the role of a single gene within a family of genes. Many small molecules interact with multiple members of a gene family confounding the validity of the intended gene as a drug target.

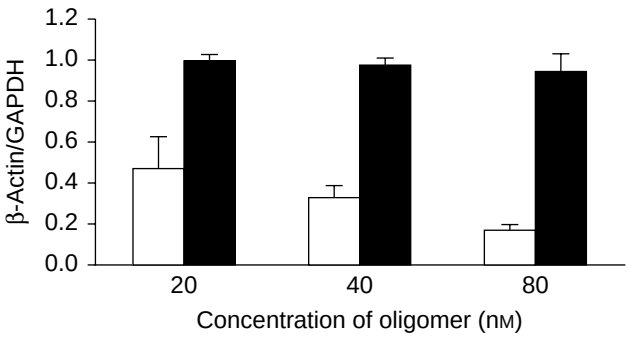


***Figure 4.*** *Concentration-dependent inhibition of β-actin mRNA by the Version D antisense oligonucleotide (ON) (Sequitur, Natick, MA, USA) S10280. The human epithelial-like A549 cells were transfected with S10280 (shown in white) and a non-targeted sequence control ON S10532 (shown in black) at concentrations of 20 nM, 40 nM and 80 nM in the presence of 2.5 μg ml$^{-1}$ Oligofectin G (Sequitur). Four hours after the start of transfection, the cells were lysed and total RNA isolated. Levels of β-actin and glyceraldehyde phosphate dehydrogenase (GAPDH, internal control) were determined by northern blot analysis, followed by densiometric analysis (Scion Image beta 3, Scion Corp., Frederick, MD, USA). Levels of β-actin-mRNA expression were normalized to the levels of GAPDH expression. Data shown represent the mean ± SD of triplicate transfections. The results indicate a concentration-dependent inhibition of β-actin mRNA by S10280 compared with S10532 (control).*

Using antisense technology, pharmaceutical companies can rapidly screen potential drug targets, and researchers can identify several lead genes that they believe are linked to a disease phenotype. Antisense ONs targeted to each of those genes can be designed and employed in a cell-based assay to rapidly evaluate the phenotypic changes resulting from target inhibition. Such experiments allow pharmaceutical researchers to quickly determine which of those genes is the best target for drug intervention. This rational approach to drug target validation will save the pharmaceutical company's time and money in the development of new therapeutics.

## Antisense for elucidation of genetic pathways and drug target validation

Advances in microarray technology have enabled the simultaneous evaluation of thousands of genes[33]. This technology allows researchers to modify the expression of one gene and to evaluate its effect on the expression of hundreds or thousands of other genes. Microarrays have already been used to systematically evaluate the genome of *Saccharomyces cerevisiae*[34]. Marton and coworkers have shown the use of DNA microarrays to validate drug targets and to screen for secondary drug target effects in yeast[35]. To determine genetic pathways, protein expression and target mRNA can be inhibited using antisense ONs, and the effects of that inhibition on the expression of thousands of genes can be assayed using microarray technology. Such evaluations will reveal gene interaction and regulatory pathways. As new genes are identified by genomic sequencing efforts, their function and association with other genes will be readily detectable. The combination of microarray and antisense technologies will play an important role in the elucidation of genetic pathways and in the understanding of the genomic organization of higher eukaryotes.

## Conclusions and future directions

The Human Genome Project has produced sequence information for a plethora of novel genes[36]. Identification of the function(s) of these genes and gaining a better understanding of the organization of the genome will play a key role in the discovery of new pharmaceuticals. With the potential to inhibit gene expression in a specific and systematic manner, antisense technology is a logical tool for use in functional genomics. Antisense ONs are easily designed, synthesized and administered, and their effects in cell culture can be screened by high-throughput analysis. Despite the limitations of first-generation antisense oligomers[37], antisense technology is coming into its own. Advances in the technology have improved the specificity with which ONs interact with their target mRNAs, enhanced the stability of the ONs and reduced the cellular toxicity associated with transfection of ONs. With careful experimentation and inclusion of appropriate controls, antisense technology represents a viable option for systematic and high-throughput determination of gene function and drug target validation.

## Acknowledgements

The authors thank Tod Woolf for helpful discussions and assistance with the preparation of this manuscript.

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## In short…

**Morpace Pharma Group Ltd** (Concord, MA, USA) has produced a report entitled *From data to drugs: strategies for benefiting from new drug discovery technologies* to help pharmaceutical companies manage technology investments and maximize returns. The main focus of the report concerns strategies for overcoming technological and organizational hurdles to realizing commercial potential of genomics and related technologies such as combinatorial chemistry, proteomics, pharmacogenomics and bioinformatics. The report notes that the potential of these technologies is huge, suggesting that by the year 2005, the potential market for genomics-based drugs for diabetes could reach $17 billion, whilst for obesity drugs, it could reach $17.5 billion.

The report aims to offer practical strategies for improving technology evaluation, acquisition and integration, analysis of the technical problems of getting from gene sequences to drug targets, and a summary of the latest technological developments and how they might affect drug discovery and development. The report also suggests advances that might be expected in the next five to ten years.

For a copy of the report, please contact Karen Partridge, Morpace Pharma Group Ltd, Concord, MA, USA. tel: +1 978 759 1000, e-mail: kpartridge@morpacepharma.com, Web: http://www.morpacepharma.com

## In short…

**Transgene** (Strasbourg, France) has entered its MVA–HPV–IL-2 product into Phase I clinical trials for the treatment of cervical cancer. This product has been designed to enhance the human immune system's ability to reject cancer cells. The product consists of the company's proprietary vaccinia virus vector based on the MVA strain carrying the genes for the antigens from the human papilloma virus (HPV) type 16 that is responsible for more than 50% of the cases of cervical cancer. The trials are taking place in the Baylor College of Medicine (Houston, TX, USA) in patients with grade 3 cervical intraepithelial neoplasia (CIN3), a pre-cancerous stage of the disease and are designed to explore its potential utility in the treatment and prevention of cervical cancer caused by HPV16.