Leveraging Antisense Technology in the Lungs
RNA-Targeting Therapies for Respiratory Diseases

Executive summary

Antisense oligonucleotides are short single-stranded DNA molecules designed to inhibit translation of a targeted gene to protein via interaction with messenger RNA. RNA interference (RNAi) is a related technology that incorporates antisense features, but employs small interfering (si)RNA molecules rather than DNA.

The lung is an attractive target for antisense oligonucleotides and siRNA technologies as drug delivery to this organ without systemic exposure is feasible. Antisense oligonucleotides directed against signaling molecules, cytokine receptors, and transcription factors involved in allergic responses have been applied in experimental models of asthma and demonstrate potential as therapeutics. Equally exciting, several antisense-based drugs directed against oncogenes have been developed for therapy of lung cancer, with some currently in late-stage clinical trials – notably Genasense® (oblimersen), an antisense oligonucleotide targeting the antiapoptotic protein Bcl-2 that is in phase II/III trials with Genta.

Although the development of antisense oligonucleotides and siRNA for therapy of lung infections is still at early stages of development, initial studies have yielded promising results against medically important pathogens such as respiratory syncytial virus. While issues of specificity, identification of correct molecular targets, drug-delivery and carrier systems, and potential adverse effects must be carefully evaluated before clinical application, antisense-based therapy is a promising new technology for lung disease that may become a commercial reality in the near future.

Preventing the production of a disease-related protein by reducing or eliminating its messenger (m)RNA is an attractive concept for clinical therapy, differing significantly from the use of traditional drugs designed to inhibit disease-related proteins already present. The two major therapeutic methods to alter mRNA levels are antisense and RNA interference (RNAi), with antisense currently being the more developed technology.

Antisense oligonucleotides (ASO) are short single-stranded DNA molecules designed to block the translation of a targeted gene to protein by interacting with mRNA. Although the concept of using antisense to specifically inhibit gene expression was first proposed almost 30 years ago,[1] 20 years passed before commercialization of the first (and currently only) antisense drug in clinical use – Vitravene® (fomivirsen; Isis Pharmaceuticals), an ASO used topically to treat cytomegalovirus-associated retinitis in patients with AIDS. RNAi is a related technology that incorporates antisense features, but employs small interfering RNA (siRNA) molecules rather than DNA and has yet to be commercialized for clinical therapy.

Despite the limited success to date, the theoretical advantages of mRNA-targeting technologies over conventional drugs see more than 30 such drug candidates currently undergoing clinical trials, with hundreds more in preclinical development and early research. The lung is an attractive target for ASO and RNAi technologies as drug delivery to this organ can be achieved relatively easily with limited systemic involvement. However, ongoing development of mRNA-targeting drugs must address several important practical issues relating to their clinical application.

Antisense technology – shooting the messenger

Despite intensive studies, the in vivo mechanism of action of ASO on mRNA is not fully understood. The current model is that ASO binding to mRNA activates the RNAse H endonuclease, resulting in mRNA degradation and inhibition of translation of a specific protein. It also appears that ASO binding to mRNA can physically block the assembly of the ribosomal complex or inhibit RNA splicing. Although this approach is conceptually simple, initial development of antisense technology was unsuccessful for two main reasons. First, oligonucleotides are large molecules that are highly negatively charged and therefore do not readily penetrate hydrophobic cell membranes; second, even if entry into cells is achieved, oligonucleotides are rapidly degraded by nuclease enzymes before they can bind target mRNA. Thus, critical issues in the development of ASO-based therapy for respiratory diseases include biological stability, delivery to the target organ and cells, and target selection and specificity.

[1] The full text of this article was published in BioDrugs 2006; 20 (1): 1-11. The original article was written by Marina Ulanova and Dean Befus from the University of Alberta, Edmonton, AB, Canada, and Alan Schreiber from the University of Pennsylvania School of Medicine, Philadelphia, PA, USA.
Phosphorothioate backbone for increased stability

To overcome the problem of oligonucleotide degradation, several chemical modifications of ASO have been developed. The first – and still most commonly used – was the phosphorothioate backbone, which comprises the replacement of a nonbridging oxygen atom by a sulfur atom at each phosphorus group. The presence of this sulfur atom substantially hinders degradation by nucleases. Following systemic administration, phosphorothioate oligonucleotides bind to plasma proteins, ensuring their prolonged effect. Several other structural modifications have also been developed, including methyloligonucleotides, morpholino, peptide nucleic acids and locked nucleic acids. Although some have improved stability against nucleases and increased binding affinity to mRNA, they can also have drawbacks such as low cell penetrance and lack of RNAse H recruitment.

Target selection and specificity

Correct target selection is critical in development of ASO-based therapy of respiratory diseases. The targeted molecule must not only be important in disease pathogenesis but, as ASO can be extremely potent, must also be specific for both the lung and the disease to avoid potential adverse effects. Once a clinically relevant target protein has been selected, specificity of the ASO is a critical issue; it must inhibit expression of the target gene but not other genes with similar sequences – i.e. the targeted mRNA sequence should not have homology to other genes. Therefore, in design of ASO, the genome should be carefully checked for possible hybridization of the ASO to sequences in nontargeted genes. Sequences common to several molecules of the same family or domains expressed in many genes must be avoided.

Delivery by cationic liposomes or surfactants

Cationic liposome complexes of ASO are often used for intravenous and local delivery of ASO to the airways, as such complexes are internalized by cells more efficiently than naked ASO. Upon systemic application for cancer therapy, ASO-liposome complexes preferentially enter tumor tissues because of increased permeability of blood vessels in tumors. In addition, cationic lipids can greatly enhance the immunostimulatory properties of DNA, providing an additional therapeutic effect that may be important in cancer therapy. However, this immunostimulation can also have adverse effects. Although carriers such as polyethylenimine (PEI) and chitosan-DNA nanospheres have been developed as alternatives to cationic liposomes, carrier-related adverse effects have seen the use of a natural surfactant with cationic properties become an attractive new method for ASO delivery to the lung.

Toxicity issues

Adverse effects of ASO therapy can result from nonspecific hybridization of ASO, nonspecific activation of RNAse H, off-target biological effects of the oligonucleotide, or from the delivery system. Nonspecific hybridization of ASO and nonspecific activation of RNAse H can each result in reduced expression of nontarget genes and consequent adverse effects. Phosphorothioate-modified oligonucleotides bind to a family of heparin-binding proteins – including certain growth factors and their receptors, and extracellular matrix proteins and adhesion molecules – which at least partially explains some of their adverse effects such as low platelet count and low blood pressure. The presence of immunostimulatory cytosine-guanine dinucleotide (CpG)
motifs within ASO is normally undesirable as they can stimulate Toll-like receptor (TLR) 9 on several cell types, and can be an important source of adverse effects related to systemic cytokine release, such as fatigue, fever and flu-like syndrome. However, in some instances they may be included because of additional beneficial effects on the immune system. Induction of pro-inflammatory cytokines can also be caused by siRNA molecules binding to TLRs present on immune cells and eliciting cellular activation.

Local delivery of ASO offers advantages over systemic administration because it allows lower doses to be used and thus minimizes systemic toxicity. An important consideration in using ASO in the airways is that adenosine can be released as an oligonucleotide degradation product and can activate adenosine receptors that induce bronchoconstriction. Indeed, adenosine receptors are up-regulated in certain clinical conditions, notably asthma (and have been targeted for ASO treatment of asthma). A further source of potential adverse effects is the delivery system, as cationic liposome complexes may enhance the immunostimulatory effects of ASO, triggering release of pro-inflammatory cytokines, or may directly affect cellular functions.

**Antisense for asthma**

As asthma is a complex heterogeneous disease involving multiple genes and poorly understood gene-environment interactions, a major challenge for ASO therapy is to identify appropriate molecular targets. It is also important to identify delivery systems that target the lung and minimize systemic distribution and related adverse effects. There are several examples of such approaches in experimental models.

**Targeting tyrosine kinases**

The tyrosine kinase SYK mediates early signaling events important in the pathophysiology of allergic asthma and initiated by cross-linking high affinity receptors for IgE on mast cells and basophils. Treatment of rats with nebulized SYK ASO-liposome complexes has been shown to inhibit SYK mRNA and protein expression in alveolar macrophages. In addition, aerosolized SYK ASO inhibited many central components of allergic asthma and inflammation in animal models of disease – such as inflammatory cell infiltration in the airways, lung eosinophilia (an increase in eosinophilic leukocytes) and the increase in tumor necrosis factor in bronchoalveolar lavage induced by antigenic challenge. SYK ASO also suppressed antigen-induced tracheal contraction.

However, although SYK is a promising molecular target for ASO therapy of asthma and other inflammatory conditions such as acute lung injury, there are potential risks related to SYK inhibition. For example, recent studies implicated SYK as a tumor suppressor gene in breast and gastric cancer. Thus, while SYK ASO may have advantages as a short-term local therapy for severe lung conditions – for example, acute respiratory distress syndrome – long-term application of SYK ASO raises potential safety issues that must be further assessed. LYN, a Src-family kinase, is another potential target for ASO therapy of asthma. LYN signaling activates SYK and is implicated in pathogenesis of asthma, although ASO therapy targeting LYN has not yet been applied in experimental models of asthma.

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**T\(_2\) pathways and other targets in inflammatory cell activation**

The use of ASO to target other points in intracellular pathways involved in inflammatory cell activation has also been investigated. For example, inhalation of ASO targeted to p38\(_{\alpha}\) mitogen-activated protein kinase (MAPK14) reduced asthma symptoms in a murine model. Similarly, intravenous administration of ASO targeting the p65 subunit (RELA) of the pro-inflammatory transcription factor nuclear factor kappa B (NF\(\kappa\)B) significantly inhibited allergic responses in a mouse model. Nevertheless, despite this proof-of-principle study, systemic administration of NF\(\kappa\)B-targeting ASO would not appear to be a feasible therapeutic strategy for asthma given the crucial involvement of this transcription factor in regulation of immune responses.

Given the important role of T helper type 2 (T\(_2\)) cytokines and their receptors in allergic asthma, various molecules involved in T\(_2\) signaling pathways have been investigated as targets for ASO therapy, including: interleukin (IL)-5, stem cell factor (KIT ligand), the transcription factor GATA-3, and the signal transducer and activator of transcription (STAT)-1. In rodent models of asthma, ASO targeting each of these mediators were found to alleviate disease symptoms. Intratracheal injection of ASO targeting the common \(\beta\) chain of IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) receptors significantly reduced disease symptoms in a rat model of asthma; this approach is moving forward to clinical testing. To directly target
bronchial smooth muscle contraction in asthma, ASO to the type 1 adenosine receptor (ADORA1) was administered in aerosol form to rabbits and found to significantly reduce both bronchoconstriction and airway inflammation. This ASO-based therapy is currently in a phase II clinical trial.

Alternative approaches

The previously described ASO approaches to asthma therapy were administered as phosphorothioate oligonucleotides, either in liposome delivery systems or as naked DNA. Recently, adenovirus-mediated intracellular expression of ASO has been employed as a delivery method, with some success in animal models of asthma. The use of recombinant adenovirus for ASO delivery to target cells offers some advantages over other methods, such as selectivity to airway epithelium and prolonged expression of transfected ASO-encoding genes (>1 week following instillation). However, adenovirus-mediated gene delivery induces immune responses to adenovirus that preclude repeated applications and there are several safety concerns such as the potential for oncogenic transformation. Other recent approaches include the use of ribozymes (RNA enzymes) and siRNA. The use of ribozymes offers some advantages over ‘traditional’ antisense because these catalytic RNA molecules can recycle after inducing the cleavage of complementary mRNA cleavage and therefore appear to act more efficiently than DNA-based antisense. A recent study employed siRNA to silence gene expression of STAT-6, a transcriptional regulator of Th2 cytokines, with subsequent reduction of certain inflammatory responses in human bronchial epithelial cells.

Antisense technology is deceptively simple in concept but in practice has proven difficult to implement. However, there are signs that the technology is finally emerging as a valid therapeutic platform.

It is important to note that in animal studies of asthma, ASO to various target molecules were applied prior to antigenic challenge. Whether or not ASO therapy will also be effective during ongoing allergic inflammation requires further studies. New targets for antisense therapy of asthma are also emerging, as genetic studies have identified several potential asthma susceptibility genes, such as inflammatory mediators, a disintegrin and metalloprotease domain 33 (ADAM33) and G-protein coupled receptor for asthma susceptibility (GPRA). Although biochemical mechanisms linking many of these candidate genes to asthma pathogenesis are poorly understood, ASO strategies may help to elucidate such pathways.

Treating lung cancer

In lung cancer, many oncogenes have been identified as targets for antisense therapy. Several antisense-based drugs have reached phase II–III clinical trials, and some may soon be approved for clinical use. Of particular promise, an ASO directed against the gene encoding the antiapoptotic protein Bcl-2, Genasense® (oblimersen), induces apoptosis of non-small-cell lung cancer cells and potentiates effects of chemotherapy. Genasense is currently undergoing phase II/III clinical trials with Genta for therapy of both small-cell and non-small-cell lung cancer in combination with chemotherapy. Other targets for antisense therapy of lung cancer that have been evaluated in clinical trials – often in combination with chemotherapy – include the signal transduction molecules protein kinase C-α, the regulatory subunit R1-α of protein kinase A, RAF kinase, and the protein encoded by the HRAS oncogene.

Generally, ASO drugs studied in clinical trials in lung cancer are short (18–26 mers) phosphorothioate oligonucleotides that, when infused intravenously, are associated with moderate dose-dependent systemic toxicity such as a decrease in platelets (thrombocytopenia), flu-like syndrome, low blood pressure and weakness. Unfortunately, there are no published reports on local application of ASO in lung cancer. Numerous preclinical studies are focused on molecular targets involved in regulation of apoptosis, cell cycle progression, angiogenesis and metastasis – including the apoptosis suppressor survivin (BIRC5), the cytochrome c oxidase assembly protein COX17, several growth factor receptors and receptor tyrosine kinases, as well as transcription factors. Although human cancer cell lines preserve their RNAi machinery, and therefore the use of siRNA to silence oncogenes involved in cancer pathogenesis has been suggested, siRNA-based strategies for lung cancer therapy remain in early stages of investigation.

Lung infections

The development of antisense-based drugs for therapy of lung infections is at an earlier stage of development than antisense programs in asthma and lung cancer. In preliminary studies, there has been some success with an antisense strategy targeting respiratory syncytial virus (RSV) in combination with the antiviral drug ribavirin. In addition, siRNA strategies targeting RSV, parainfluenza virus, and the recently discovered coronavirus SARS-CoV...
Table I. Biopharma companies with siRNA programs for respiratory diseases

| Company                | Indication       | Target                                         | Development status | Comments                                                                 |
|-----------------------|------------------|-----------------------------------------------|--------------------|--------------------------------------------------------------------------|
| Alnylam Pharmaceuticals| Respiratory syncytial virus (RSV) | RSV nucleocapsid (N) gene                      | Phase I (US, Europe) | Intrasinally administered; product code ALN-RSV01                         |
|                       | Influenza        | Genes conserved across all influenza strains  | Preclinical        | In collaboration with Novartis; focusing on pandemic flu strains; Investigational New Drug filing anticipated by end 2006 |
| Sirna Therapeutics    | Asthma           | T_{h}2-type cytokines                         | Preclinical        | Aerosolized formulations; clinical studies anticipated in 2006          |
| Nastech Pharmaceutical | Influenza        | Viral genes                                   | Preclinical        |                                                                          |

a Source: R&D Insight (Adis International).

siRNA = small interfering RNA; T_{h}2 = T helper type 2.

– the causative agent of severe acute respiratory syndrome (SARS) – have shown promising results in animal studies. These efforts signal the beginning of a potentially important research area, with opportunities to develop innovative ASO therapies for infectious diseases of the lung.

**Advancing antisense for lung disease**

Antisense technology is deceivingly simple in concept but in practice has proven difficult to implement as pharmacotherapy. However, there are signs that the technology is finally emerging as a valid therapeutic platform, with various ASO-based drug candidates showing promise for treatment of respiratory diseases such as asthma, lung cancer and viral infection.

Applying ASO in a complex heterogeneous disease such as asthma presents a major challenge, since this condition involves several pathways, numerous genes and poorly understood gene-environment interactions. Despite this, some approaches to specifically target critical molecules in asthma have been successful and development of ASO-based drugs for clinical application can be anticipated in the near future. These efforts will be aided greatly by the rapidly developing field of asthma genetics. ASO therapy also has the potential to become a powerful tool against lung cancer. Successes are anticipated based on intensive molecular studies, discovery of causal oncogenes in lung cancer, and ASO drug candidates in late-stage clinical trials. Although application of RNAi has unique challenges, several companies have programs developing siRNA-based therapies for respiratory diseases (table I).

Ongoing critical challenges in the development of ASO-based therapeutics include selection of the correct target protein, and minimization of sequence-specific (e.g. CpG-mediated) and sequence-nonspecific (e.g. phosphorothioate-mediated) adverse effects. New methodologies for delivery of ASO to selected target cells and optimization of carrier systems are likely to provide important advances for therapeutic uses of ASO.

**Reference**

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