A review of antisense therapeutic interventions for molecular biological targets in asthma

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Abstract: Modern tools of genomics and proteomics reveal potential therapeutic antisense targets in asthma, increasing the interest in the development of anti-mRNA drugs. In allergic asthma experimental models, antisense oligonucleotides (ASO) are administered by inhalation or systemically. ASO can be used for a large number of molecular targets: cell membrane receptors (G-protein coupled receptors, cytokine and chemokine receptors), membrane proteins, ion channels, cytokines and related factors, signaling non-receptor protein kinases (tyrosine kinases, and serine/threonine kinases) and regulators of transcription belonging to Cys4 zinc finger of nuclear receptor type or beta-scaffold factors with minor groove contacts classes/superclasses of transcription factors. A respirable ASO against the adenosine A_1 receptor was investigated in human trials. RNase P-associated external guide sequence (EGS) delivered into pulmonary tissues represents a potentially new therapeutic approach in asthma as well as ribozyme strategies. Small interfering RNA (siRNA) targeting key molecules involved in the patho-physiology of allergic asthma are expected to be of benefit as RNAi immunotherapy. Antagomirs, synthetic analogs of microRNA (miRNA), have important roles in regulation of gene expression in asthma. RNA interference (RNAi) technologies offer higher efficiency in suppressing the expression of specific genes, compared with traditional antisense approaches.

Keywords: asthma, antisense oligonucleotides, ribozymes, RNA interference

Introduction

Asthma is one of the most common chronic diseases, throughout the world being estimated at around three hundred million asthmatic persons. Global Initiative for Asthma guidelines are currently revised. These international recommendations are based on scientific evidence elicited from therapeutic interventions (Bousquet et al 2007), and periodic assessment of asthma control is considered more relevant and useful (Humbert et al 2007). Asthma is a complex chronic inflammatory disorder of the airways, usually allergic, characterized at molecular level by upregulated expression of genes encoding multiple inflammatory proteins, such as cytokines and chemokines, adhesion molecules, mediator-synthesizing enzymes, proinflammatory receptors, and dysregulated apoptosis in some inflammatory cells.

The mechanism of action for conventional asthma drugs usually involves binding to different proteins or interfering with their activity, these therapies only temporarily preventing targeted protein function. Standard asthma treatments can be classified as controllers and relievers. *Relievers* are drugs used on an as-needed basis that reverse bronchoconstriction and relieve its symptoms (rapid acting inhaled selective $beta_2$-agonists). *Controllers* are drugs taken on a long-term basis to keep asthma under clinical control mainly through their anti-inflammatory effects (glucocorticosteroids, antileucotrienes). Inhaled corticosteroids are the most effective controller drugs currently available. These drugs with potent anti-inflammatory effects can be regarded as agonists of Cys4 zinc finger transcription factors because they activate

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the glucocorticoid receptor for the direct or indirect regulation of expression of target genes (Popescu 2005).

Although there is no cure for persistent asthma, appropriate management often results in the achievement of control, but requires daily medication, which may reduce compliance. Moreover, current therapies fail to restore the immunological imbalance, frequently do not produce an optimal control of asthma symptoms, and sometimes are associated with adverse effects.

Despite significant advances that have been made in recent years, there is still an urgent need for novel, more effective and safer asthma drugs. An important objective in molecular pharmacology is the manipulation of gene expression with new drug molecules. RNA-based gene silencing strategies have been proposed not only as research tools, but also as potential therapeutic interventions in allergic asthma.

Post-transcriptional inhibition of gene expression at the mRNA level can be accomplished using antisense oligonucleotides (ASO), DNAzymes, ribozymes (RZ), RNase P-associated EGS molecules (EGS), and, more recently, interference RNA (RNAi) manipulation, including small interfering RNA (siRNA). Concerted efforts from both academia and research-driven pharmaceutical companies have made significant progress in turning these nucleic acid drugs into therapeutics (Mahato et al 2005; Pan and Clawson 2006).

A review of the studies regarding antisense approaches in asthma is important as an integrated framework for the patho-physiological rationale and potential clinical application of biologic anti-mRNA agents. Animal models of allergic asthma, especially those involving rodents and nonhuman primates, are necessary for the development of such therapies (Isenberg-Feig et al 2003). Mouse, guinea pig and rat asthma models are still used in many experiments. Disadvantages must be considered, including difficulties related to the techniques for measuring pulmonary function, and species differences in expression or responses to pro-inflammatory mediators. Large mammal models of allergic asthma have been developed in rabbits, dogs, cats, sheep, monkeys (rhesus and cynomolgus), primate models being much closer to human persistent asthma. Antisense therapies with nucleic acids that specifically target the human immune system are not properly active in animals less genetically similar to man (Coffman and Hessel 2005). Accelerated characterization of asthma-relevant molecular targets created increasing interest in the development of antisense agents, that could significantly reduce the duration of an anti-asthma drug development process.

The modern tools of genomics and proteomics reveal potential therapeutic antisense targets: membrane and intracellular receptors, channels, transporters, enzymes, a multitude of factors and regulators (cytokines, chemokines, growth factors, transcription factors etc), structural proteins, and nucleic acids etc. Theoretically, any protein can be targeted for antisense intervention. Although receptors and cytokines are frequently chosen targets for anti-mRNA agents, various classes of signal transduction molecules are also paid special attention (Popescu 2005a).

Biological targets for new therapeutic interventions in asthma must have some characteristics: critical and preferably unreplaceable roles in asthma pathogenesis, a certain level of functional and structural novelty to allow drug specificity, insignificant involvement in other important biological processes (in order to limit potentially serious side effects), a constrained level expression or tissue selectivity to allow sufficient drug efficacy (Zheng et al 2006).

Antisense oligonucleotides (ASO)

ASO are short single-stranded synthetic nucleic acid polymers, consisting of 15–25 nucleotides, that induce the inhibition of target gene expression by exploiting their ability to bind to the target messenger RNA (mRNA) by Watson-Crick base-pairing. The antisense effects (sequence-specific effects) of ASO are mainly due to the hybridization with the target mRNA in a sequence-dependent complementary manner (binding is performed *via* hydrogen bonds).

First generation ASO have a phosphorothioate backbone modification with replacement of non-bridging oxygen atoms in the phophodiester bond substituted by sulfur, in order to increase resistance to nucleases. The most important mechanism of action of phosphorothioate ASO is the activation of RNase H, which cleaves the RNA moiety of a DNA-RNA heteroduplex, leading to the degradation of the targeted mRNA, and thus preventing translation of the specific protein. ASO binding to mRNA can also inhibit translation by steric blockade of the ribosome (inhibition of the ribosomal complex formation) or interfere with mRNA maturation by inhibiting RNA splicing (Kurreck 2003; Chan et al 2006; Ulanova, Schreiber, et al 2006).

A disadvantage of phosphorothioate ASO is their binding to the basic fibroblast growth factor (a member of the heparin-binding family of growth factors), inhibiting its binding to cell surface receptors, and removing it from low affinity binding sites on extracellular matrix (Guvakova et al 1995). Such mechanisms may be involved in some adverse effects of systemic ASO delivery (Ulanova, Schreiber, et al 2006). A dose-limiting toxicity aspect is represented by reversible thrombocytopenia related to the phosphorothioate backbone

(Cotter 1999). Hypotension associated with complement activation, which can result due to binding to various proteins and the polyanionic nature of ASO (Ulanova, Schreiber et al 2006), are possible in primates (Sereni et al 1999).

Immunostimulatory unmethylated deoxycytosine-deoxyguanosine (CpG) motifs are normally undesirable as they can stimulate Toll-like receptor 9 (TLR9) on several cell types, and produce Th1-like proinflammatory cytokine release, with fever and flu-like syndrome (Klinman 2004). Many phosphorothioate ASO contain CpG dinucleotides, and their position and the sequences flanking play critical role in determining immunostimulatory activity (Agrawal and Kandimalla 2004). Hepatotoxicity may be also related to CpG motifs. Non-specific biological effects are possible results of four contiguous guanine residues, the guanine quartets (Ulanova, Schreiber et al 2006).

Second generation ASO contain nucleotides with alkyl modifications at the 2′ position of the ribose. 2′-*O*-methoxyethyl-oligonucleotides (2′MOE-ASO) have increased binding affinity to mRNA and resistance to degradation. Because they do not recruit RNase H, the antisense effect can be due to the steric blockade of translation. Non-CpG-containing 2′MOE-ASO have a lower proinflammatory risk, independent of TLR9 (Senn et al 2005).

Other compounds with alternative structures form a third generation of ASO, including peptide nucleic acids, morpholino oligonucleotides, locked nucleic acids, etc (Kurreck 2003). Because of low cell penetrance of many of these new generation ASO, first generation representatives are still commonly used (Ulanova, Schreiber et al 2006). Recently, two different configurations of chimeric ASO (altimers and gapmers) containing 2′-deoxy-2′-fluoro-β-D-arabinonucleic acid (FANA) were found to have potent antisense activity (Ferrari et al 2006).

In experiments with ASO, there is a need of differentiation between the desired sequence-specific inhibition of the targeted mRNA from the undesired sequence-related and non-sequence-related effects. Negative controls serve to rule out the possibility that ASO effects are caused by non-sequence-specific mechanisms, while positive controls provide additional evidence that a true antisense effect is the reason for the biological effects. A combination of controls is recommended. Sense controls are oligonucleotides having a sequence complementary to that of the ASO (identical to the mRNA target), while reverse controls are obtained by reversing the antisense sequence with respect of its 5′–3′ orientation. Random (nonsense or scrambled) controls are generated by mixing up the ASO bases in a randomized manner, while mismatch controls are obtained by the deliberate introduction of one or more mismatches into the antisense sequence (Brysch 2000).

The pharmacokinetics of ASO depends on the system and mode of delivery. First generation ASO bind to plasma proteins after systemic administration, ensuring a prolonged effect. Local delivery of ASO to the airways (respirable ASO) is effective at smaller doses compared with other routes of administration. ASO delivered as aerosols directly to the lungs, minimize the potential for non-antisense systemic side effects and toxicity (Tanaka and Nice 2001). *In vivo* absorption, tissue distribution, metabolism and excretion, efficacy and safety studies of respirable ASO suggest that these antisense agents can be safely delivered to target respiratory tissues in low, but effective doses (Ali et al 2001; Sandrasagra et al 2002; Popescu 2003).

Enhancing the delivery of ASO molecules to cells is an essential issue for antisense therapeutics. Potential adverse effects may also be related to ASO delivery systems. Cationic liposome formulations, used as transmembrane carriers, enhance immunostimulatory properties of ASO with systemic pro-inflammatory cytokine release and may affect cellular functions directly (Ulanova, Schreiber et al 2006), by inhibiting TNF-alpha-induced endothelial vascular cell adhesion molecule-1 (VCAM-1) expression in human pulmonary artery endothelial cells (Maus et al 1999). Although not as potent *in vitro* as cationic lipid systems, cell-penetrating peptides-oligonucleotide conjugates may have substantial advantages *in vivo* over particle-based delivery (Juliano 2006).

In the treatment of allergic asthma, ASO can be used for silencing of gene expression, at post-transcriptional level, for many molecular targets: cell membrane receptors (G-protein coupled receptors, cytokine and chemokine receptors), membrane proteins, ion channels, cytokines and related factors (Table 1), signaling non-receptor protein kinases (tyrosine kinases, such as Syk, and serine/theonine kinases, such as p38 MAP kinase) and regulators of transcription belonging to Cys4 zinc finger of nuclear receptor type (GATA-3) or beta-scaffold factors with minor groove contacts (p65, STAT-6) classes/superclasses of transcription factors (Table 2) (Popescu 2005a).

Aerosolized adenosine A_1 receptor ASO (EPI-2010) is a respirable phosphorothioate ASO targeting adenosine A_1 receptor (AA1R) abnormally overexpressed in the allergic lung (Ali et al 2001). The gene ADORA1 located on chromosome 1q32.1 encodes AA1R that belongs to the G-protein coupled receptor 1 family. AA1R ASO reduced adenosine A_1 receptors number in bronchial smooth muscle cells, and

Table 1 ASO targeting cell surface receptors, membrane proteins, ion channels, cytokines and related factors in allergic asthma

Molecular target of ASO-based therapy	Gene name [chromosomal location]	ASO route of administration in the experimental model (reference)
Adenosine A_1 receptor (AA1R)	ADORA1 [1q32.1]	AA1R ASO – inhalation house-dust mite-sensitized rabbits, *Ascaris*-sensitized primate asthma models, humans with asthma (Nyce 2002)
IL-5 receptor alpha chain (IL-5R-alpha, CD125)	IL5RA [3p26–p24]	IL-5R-alpha ASO – intravenous ragweed-induced allergic peritonitis murine model (Lach-Trifilieff et al 2001)
GM-CSF/IL-3/IL-5 receptor common beta-chain (CD131)	CSF2RB [22q12.2–q13.1]	GM-CSF/IL-3/IL-5 R-beta ASO – intratracheal rat allergic asthma model (Allakhverdi et al 2002)
C-C chemokine receptor type3 (CCR-3)	CCR3 [3p21.3]	CCR-3 ASO (ASA4) – intratracheal simultaneously with GM-CSF/IL-3/IL-5 R-beta ASO rat allergic asthma model (Allakhverdi 2006)
alpha-4 subunit of VLA-4 receptor (CD49d)	ITGA4 [7p12.3–p12.1]	CD49d ASO (ISIS 107248) – inhalation OVA-challenged mouse lung (Lofthouse et al 2005)
CD86 (B7-2)	CD86 gene [3q21]	CD86 ASO – inhalation OVA-induced mouse model of asthma (Crosby et al 2007)
Gob-5 (Ca2+-dependent chloride channel-1)	CLCA1 [1p31–p22]	Gob-5 ASO – intratracheal (adenoviral vector) mouse model of asthma (Nakanishi et al 2001)
interleukin 4 (IL-4)	IL4 [5q23–q31]	IL-4 ASO treated CD4+ cells from OVA-sensitized rats, administered intraperitoneally to naive rats challenged later with OVA aerosol (Molet et al 1999)
interleukin 5 (IL-5)	IL5 [5q23–q31]	IL-5 ASO (ISIS 20391) – intravenous murine OVA-induced asthma and allergic peritonitis model (Karras et al 2000)
Kit ligand/stem cell factor (SCF)	KITLG [12q22]	SCF ASO – intranasal OVA-sensitized mice with asthma (Finotto et al 2001a)
Tumor necrosis factor-alpha (TNF-alpha)	TNFA [6p21.3]	TNF-alpha AS-ON (ISIS 104838) – inhalation mouse model of chronic asthma (Crosby et al 2005)

Abbreviations: ASO, antisense oligonucleotide; OVA, ovalbumin; VLA-4, very late antigen-4; OVA, ovalbumin.

preclinical studies in rabbits and primates revealed that EPI-2010 attenuates adenosine- and allergen-induced bronchial obstruction. The ability of EPI-2010 to block airway hyperresponsiveness (AHR) to inhaled adenosine persisted for several days, giving it the potential to be a once-*per*-week treatment for asthma (at the time of phase I clinical trials). The effect on the inflammatory markers from bronchoalveolar lavage (BAL) and lung immunohistopathology were not reported. A single effective dose was 50 μg/kg and the effect duration was of almost 7 days (Tanaka and Nyce 2001). EPI-2010 was safe and well tolerated, but Phase IIa clinical trials revealed modest data of efficacy in patients with mild asthma (Ball et al 2003; 2004). The evidence suggests that the treatment with AA1R ASO failed to confirm the anticipated effectiveness because the pathophysiology of allergic asthma is far more complex, involving a large number of immunological changes, not only a single receptor with proinflammatory and bronchoconstrictive actions (Popescu 2005a).

Interleukin-5 (IL-5) is critical for producing specific tissue eosinophilia in allergic asthma. IL-5 receptor alpha chain (IL-5R-alpha, CD125) is encoded by the gene IL5RA (3p26–p24). ASO against IL-5R-alpha is a 2′MOE-type that decreased *in vitro* mRNA and protein expression, and *in vivo* inhibited the development of blood and tissue eosinophilia, in a ragweed-induced allergic peritonitis murine model (Lach-Trifilieff et al 2001). GM-CSF/IL-3/IL-5 receptor common beta-chain (CD131) is encoded by CSF2RB (22q12.2–q13.1). GM-CSF/IL-3/IL-5 R-beta ASO (AS143), a phosphorothioate ASO administered intratracheally in a rat allergic asthma model, reduced lung beta chain of IL-3/IL-5/GM-CSF mRNA and protein expression, and inhibited antigen-induced lung eosinophilia and AHR to LTD_4 (Allakhverdi et al 2002).

C-C chemokine receptor type 3 (CCR-3) is the main chemokine receptor involved in eosinophil attraction into airway inflammation in asthma. A multitargeted approach with the blocking of multiple inflammatory pathways

Table 2 ASO targeting non-receptor protein kinases and transcription factors in allergy

Molecular target of ASO-based therapy	Gene name [chromosomal location]	ASO route of administration in the experimental model (reference)
Spleen tyrosine kinase (Syk)	SYK [9q22]	Syk ASO – inhalation (ASO:liposome complexes) infectious model of airway inflammation induced by *Nippostrongylus brasiliensis* larvae and IgE-mediated OVA-induced asthma in rat (Stenton et al 2000; 2002; Ulanova et al 2003)
Mitogen-activated protein kinase 14 (p38 alpha MAP kinase)	MAPK14 [6p21.3–p21.2]	p38 alpha MAPK ASO (ISIS 101757) – inhalation or nose-only exposure OVA-sensitized mice with asthma (Duan et al 2005)
p65 subunit of NF-kB (p65 subunit nuclear factor NF-kappaB)	RELA [11q13]	p65 ASO – intravenous OVA-sensitized mice with asthma (Choi et al 2004)
GATA-3 (a T-cell specific transcription factor)	GATA3 [10p15]	GATA-3 ASO – intranasal OVA-sensitized murine model of asthma (Finotto et al 2001b)

Abbreviations: ASO, antisense oligonucleotide; OVA, ovalbumin; MAP kinase, mitogen-activated protein kinase.

achieved by simultaneously intratracheal administration of two ASO, AS143 and CCR-3 ASO (ASA4), in sensitized Brown Norway rats, resulted in fewer eosinophils detected in BAL of animals treated with both ASO as compared to each ASO alone, and AHR to LTD_4 significantly decreased at lower doses in the two ASO-treated groups *versus* to a single ASO (Allakhverdi et al 2006).

VLA-4 (very late antigen-4) is the $alpha_4beta_1$ integrin localized on many inflammatory cells that participates in cell adhesion, trafficking and activation, through binding to VCAM-1 and fibronectin. Small molecule VLA-4 inhibitors have demonstrated inhibition of AHR and lung inflammation in animal asthma models. The VLA-4 alpha subunit (CD49d) may be target for second generation ASO (ISIS 107248), also known as ATL-1102. Loe dose of 2′MOE-ASO delivered by inhalation in ovalbumin (OVA)-challenged mouse lung inhibited allergen-induced eosinophil lung infiltration, mucus production, and AHR to methacholine (Lofthouse et al 2005).

Inhaled CD86 ASO may have utility in asthma treatment. The B7-family molecule CD86, expressed on the surface of pulmonary antigen presenting cells, is a type I membrane protein that is a member of the immunoglobulin superfamily. The CD86 gene is located on chromosome 3q21. CD86–CD28 signaling is involved in priming allergen-specific T cells. In an OVA-induced mouse model of asthma, inhaled aerosolized CD86 ASO suppressed OVA-induced up-regulation of CD86 protein expression on pulmonary dendritic cells, macrophages and recruited eosinophils, correlated with decreased methacholine-induced AHR, pulmonary inflammation, mucus production and BAL eotaxin levels (Crosby et al 2007).

Gob-5, a member of Ca^{2+}-dependent chloride channel family (gene CLCA1 on chromosome 1p31–p22) is an important molecule in murine asthma. Intratracheal administration of adenovirus-expressing antisense gob-5 RNA in a mice model of asthma inhibited gob-5 expression, prevented goblet cell hyperplasia, decreased AHR and mucus overproduction (Nakanishi et al 2001).

Interleukin-4 (IL-4) has important pro-inflammatory actions in asthma including induction of the IgE isotype switching, expression of VCAM-1, increase production of eotaxin by fibroblasts, epithelial and endothelial cells, and differentiation of Th_2 lymphocytes. The IL-4 receptor alpha chain plays special roles in allergy, being a common subunit of the IL-4 and IL-13 receptor complex. Signaling through it by IL-4 is important in Th_2 differentiation, and the blocking of its production inhibits the activity of IL-4 and IL-13, regulating allergic inflammation, mucus overproduction, and AHR. The IL-4 receptor alpha chain was studied as a target for new inhaled second generation ASO (ISIS 369645) (Popescu 2005).

IL-4 ASO treated CD4+ cells from OVA-sensitized rats, transferred to naive rats, decreased IL-4 expression, inhibited the adoptively transferred late phase allergic response in a dose related manner (Molet et al 1999). Genes IL4 and IL5 are located on chromosome 5q23–q31. IL-5 ASO, a 2′MOE-ASO (ISIS 20391), administered intravenously in a murine OVA-induced asthma and an allergic peritonitis model, inhibited IL-5 mRNA and IL-5 protein expression, reduced antigen-induced lung eosinophilia and inhibited the late phase AHR (Karras et al 2000).

The Kit ligand or stem cell factor (SCF) acts as an important growth factor for human mast cells, induces chemotaxis

and survival of the mast cell and enhances antigen-induced degranulation of human lung-derived mast cells. SCF gene, KITLG, is located on chromosome 12q22. SCF ASO, a fluorescein isothiocyanate-labeled phosphorothioate ASO, delivered in OVA-sensitized mice with asthma, inhibited SCF expression in interstitial lung cells, reduced IL-4 concentration in the BAL fluid, decreased eosinophil and mast cell counts in lung interstitial tissue and/or BAL fluid, as effective as a corticosteroid treatment (Finotto, Buerke, et al 2001).

TNF-alpha (tumor necrosis factor-alpha) is involved as a causative factor of allergic asthma inflammation, being a cytokine involved in many effects associated with airway epithelium (alteration of permeability, up-regulation of ICAM-1 expression, stimulation of IL-6, IL-8 and GM-CSF production) and other relevant effects in the lungs. TNF-alpha ASO (ISIS 104838), a 2′MOE-ASO, delivered by inhalation in a mouse model of chronic asthma, reduced TNF-alpha protein in BAL fluid, inhibited recruitment of eosinophils and neutrophils to the airways, decreased lung inflammation, and reduced mucus production and AHR (Crosby et al 2005).

Lyn is a member of the Src family of nonreceptor tyrosine-protein kinases that is expressed in the hemopoietic cells in asthmatic airway inflammation (with the exception of T lymphocytes). Lyn is implicated in many signaling pathways, including those for IgE, IL-5 and other cytokines and growth factors. However, the role of Lyn in asthma is controversial. Lyn ASO experiments with purified eosinophils and OVA-sensitized murine bone marrow cells, revealed that this ASO kinase blocked Lyn expression in eosinophils, prevented IL-5 receptor cascade activation and inhibited eosinophil differentiation from stem cells, attenuated antiapoptotic IL-5 effect on eosinophils, similarly to Raf-1 ASO, which in contrast inhibited also eosinophil cationic protein (ECP) secretion (Pazdrak et al 1998; Stafford et al 2002). Recently, it was reported that Lyn-deficient mice develop severe, persistent asthma, Lyn being a negative regulator of Th_2 immunity (Beavitt et al 2005).

The spleen tyrosine kinase (Syk) is an interesting new therapeutic approach in allergic asthma. This non-receptor tyrosine-protein kinase (Ulanova et al 2003) plays a wide role in the regulation of immune and inflammatory responses (Duta et al 2006), being involved in immunoreceptor signalling complexes in leukocytes, and in various signalling cascades including those originating from integrin and cytokine receptors (Ulanova et al 2005). A 60 base pairs ASO constructed as a stem-loop structure and containing three phosphorotioate modifications, for specific inhibition of Syk, was constructed. Syk ASO was delivered *in vivo* as aerosols using liposome complexes combining cationic 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) with a neutral carrier lipid, dioleoylphosphatidyl-ethanol-amine (DOPE). DOTAP-DOPE liposomes form complexes with ASO at a 2.5:1 ratio (Stenton et al 2000). Two animal models were used, an infectious model of airway inflammation induced by the helminth *Nippostrongylus brasiliensis* larvae and an IgE-mediated OVA-induced asthma in Brown Norway rats. Syk ASO reduced Syk mRNA and protein expression in inflammatory cells, depressed up-regulation of some adhesion molecules (ICAM-1, $alpha_4$ and $beta_2$ integrins), suppressed antigen-mediated trachea contraction, inhibited nitric oxide and TNF release from alveolar macrophages and antigen-induced pulmonary inflammatory cell infiltrate, decreasing eosinophilia and neutrophilia (Stenton et al 2002). Although Syk ASO significantly reduced lung inflammatory responses in experimental asthma and acute lung injury models, long term delivery must be further assessed (Ulanova, Schreiber, et al 2006).

p38 MAP kinase is a non-receptor proline-directed serine/threonine kinase with a pivotal role in the activation of inflammatory cells. p38 alpha MAP kinase ASO (ISIS 101757) is a 2′MOE-ASO delivered as aerosol for inhalation or nose-only exposure, in OVA-sensitized mice with asthma. It inhibited p38 alpha mRNA and protein expression in BAL fluid cells and peribronchial lymph node cells, reduced mucus hypersecretion, suppressed Th_2 cytokine production (IL-4, IL-5 and IL-13 levels in BAL fluid), and inhibited airway eosinophilia and AHR (Duan et al 2005).

The transcription factor, nuclear factor NF-*kappa*B (NF-κB) transactivates various genes for proinflammatory cytokines and many other immunoregulatory genes, having a important role in the regulation of airway inflammatory process in asthma. In most cells, the NF-κB prototype is a heterodimer composed of a 50 kDa (p50) and 65 kDa (p65) polypeptides. The p65 subunit is responsible for the strong transcription activating potential of NF-κB. Mice sensitized and challenged with OVA were intravenously administered p65 ASO. This supressed the allergen-specific IgE response, reduced eosinophil number, TNF-alpha and Th_2 cytokines, IL-4 and IL-5, in BAL fluid, and inhibited eosinophilic infiltration and AHR (Choi et al 2004).

GATA-3, a trans-acting T-cell specific transcription factor, encoded by the gene GATA3 (10p15), has a critical role in Th_2 differentiation (IL-4 dependent and IL-4 independent) and in Th_2 cell proliferation. GATA-3 is identified as a cell lineage-specific transcription factor selectively

expressed and activated in the Th_2 lineage as a consequence of STAT-6 activation (Finotto and Glimcher 2004). EPI-30051 is a respirable ASO targeting GATA-3 transcription factor (Paterson 2001, Popescu 2005b). GATA-3 ASO, synthesized with a phosphorothioate backbone to improve resistance to endonucleases, has the potential advantage of simultaneously down-regulation of various Th_2 cytokines expression, rather than suppressing a single mediator. A fluorescein isothiocyanate-labeled GATA-3 ASO delivered intranasal in an OVA-sensitized murine model of asthma, reduced intracellular GATA-3 lung expression due to DNA uptake in lung cells, suppressed Th_2 cytokine production, and inhibited eosinophilic infiltration and AHR (Finotto, De Sanctis et al 2001).

The new ASO of a ribbon type (RiASO) consist of two loops containing multiple antisense sequences and a stem connecting the two loops. Three antisense sequences targeting different binding sites are placed in a loop (Moon et al 2000). Ri-ASO for TGF-beta1 was recently developed (Choi et al 2005). RiASO are more stable against nucleases (Moon et al 2000) and may be used for developing antisense drugs.

A different strategy to inhibit target gene expression based on DNA technology is represented by decoy oligodeoxynucleotides (ODN). These agents are short double-stranded synthetic ODN molecules that contain transcription factor binding sites which are highly useful in transcriptional regulation. Delivered into cells, decoy ODN agents bind to nuclear transcription factors, preventing their binding to consensus sequences in target genes. Decoy ODN with a circular dumbbell structure were also developed in order to exhibit higher resistance to nucleases (Lee et al 2003; Gambari 2004).

Decoy ODN specific for transcription factor STAT-1 inhibits allergen-induced airway inflamamtion and airway hyperreactivity in a murine model of asthma (Quarcoo et al 2004). Experiments performed *in vitro* revealed targeted disruption of STAT-6 DNA binding activity by a decoy ODN blocks IL-4-driven Th_2 cell response (Wang et al 2000) and abolishing of induced IL-5 gene expression by a GATA-3 decoy ODN (Nakamura and Hoshino 2005).

Locations of previously discussed susceptibility genes for asthma, as well as associated phenotypes such as bronchial hyperresponsiveness and atopy, have been identified by genome-wide linkage analysis (Howard et al 2003). Recently some candidate genes were identified by positional cloning: ADAM33, a disintegrin and metalloprotease domain 33 (20p13), AAA1, asthma-associated alternatively spliced gene 1 (7p14.3) and NPSR1, neuropeptide S receptor 1, also known GPRA, G-protein-coupled for asthma susceptibility (7p14.3).

ADAM33 and NPSR1, strong candidates for involvement in asthma pathogenesis, may be also new targets for antisense therapy (Ulanova, Schreiber et al 2006). Antisense deletion of ADAM33 in human altered airway fibroblasts inhibits the expression of contractile proteins, such as alpha-actin, induced by TGF-beta and accelerates their apoptosis, explaining why this gene is involved in bronchial hyperresponsiveness and the progression of asthma (Holgate et al 2004). Although not every gene relevant to asthma is likely to represent a drug target for ASO, some new oligonucleotides have a potential to improve therapeutic benefits (Popescu 2005a).

Ribozymes (RZ)

RZ are synthetically engineered, catalytically active RNA molecules, capable of cleaving target mRNA (RNA enzymes), with high sequence specificity. Allergic asthma represents a clinical area suitable for RZ use (Popescu 2005a).

The most advanced in therapeutic applications are hammerhead RZ, presenting a catalytic core that cleaves any NUH triplets (where N can be any ribonucleotide, and H can be any ribonucleotide except guanosine), and two helices that bind the target mRNA. Hairpin RZs usually cleave after BNGUC (where B can be any nucleotide except adenosine). A new type of binary hammerhead ribozymes with improved catalytic activity and nucleolytic stability was recently assessed (Vorobjeva et al 2006).

To date, many protein molecules involved in asthma immunopathogenesis have been identified, and it is anticipated that the list will grow. RZ can be used to cleave the mRNA encoding such specific targets (Freelove and Zheng 2002). Hammerhead and hairpin RZs targeting conserved sequences within IL-5, NF-κB and ICAM-1 mRNA have been already designed. Pharmacokinetic studies in mice revealed that intratracheal RZs administration increase exposure in lungs (Sandberg et al 2001). Finally, it has to be taken into account that RZ may be less effective than RNAi in many respects (Li et al 2007).

RNase P-associated external guide sequence (EGS)

RNA in a complex with another oligoribonucleotide known as an external guide sequence (EGS) can become a substrate for ribonuclease P (Yuan and Altman 1994). EGS technique can be used to cleave target mRNA by forming a tRNA-like structure.

Considering that no adverse effects related to the loss of IL-4 function were observed in human subjects at pulmonary level or systemically, an EGS targeting the human IL-4 receptor alpha mRNA was recently studied in *in vitro* and tissue cultures. The gene for IL-4 receptor alpha chain (IL-4R-alpha, CD124) is IL4R (IL4RA), located on chromosome 16p11.2–p12.1.

Based on the similarity to the tRNA cleavage consensus sequence and location in exposed regions of the human IL-4 receptor alpha mRNA, the sequence EGS1 was designed (Dreyfus et al 2004). This efficiently directs RNase P-mediated cleavage of mRNA for human IL-4R mRNA. Blockade of the IL-4 receptor alpha chain is able to inhibit the pathogenic effects of both IL-4 and IL-13 using a single antisense agent.

Although no trials of EGS have been reported in human or animal models, it is possible that inhaled small nuclease-resistant EGS delivered into pulmonary tissues using endogenous lung surfactants may represent a potential new therapeutic approach in asthma. Because of the potential for a single EGS to recycle and due to different mechanisms of action, involving the ability to act as a co-catalyst for the ubiquitous RNase P, inhaled EGS could have advantages over conventional ASO. Reduced off-site cleavage by EGS is an advantage, not only over conventional ASO, but also relative to RNAi (Dreyfus et al 2004). These data suggest that the effect of EGS is more rapidly apparent compared with that of siRNA (Zhang and Altman 2004). Another advantage is represented by the fact that RNase P is ubiquitously present in cells, in significant amounts (Dreyfus et al 2004).

RNA interference (RNAi)

After the discovery of RNA interference and its mechanisms (Fire et al 1998; Mello and Conte 2004), it was considered that RNAi might be a useful approach in future gene therapy (Soutschek et al 2004; Zimmermann et al 2006). RNAi is an endogenous gene-silencing mechanism that involves double-stranded RNA-mediated sequence-specific mRNA degradation. RNAi is an evolutionarily conserved post-transcriptional RNA-dependent gene silencing mechanism. RNAi pathways are guided by small non-coding RNA molecules, such as double-stranded small interfering RNA (siRNA) and microRNA (miRNA), key regulators of gene expression. Both siRNA and miRNA are loaded into the RNA-induced silencing complex (RISC) where they guide mRNA degradation or translation repression dependent on target complementarity (Figure 1 represents a scheme of the mechanism of action of antisense therapy strategies). RISC may be an attractive target for novel pharmacological therapies (Dreyfus 2006).

The RNAi pathway is initiated by the Dicer enzyme, a riboendonuclease belonging to the RNase III family, that cleaves double-stranded RNA (dsRNA) and pre-miRNA into short double-stranded RNA fragments about 20–25 nucleotides long, usually with a two-base overhang on the 3′ end. The short RNA fragments are known as siRNA when they derive from exogenous sources and miRNA when they are produced from pre-miRNA. Processed from pri-miRNA by the RNase III Drosha and its partner dsRNA-binding protein Pasha, pre-miRNA are stem-loop precursors of approximately 70 base pairs transported into the cytoplasm by exportin 5.

dsRNA and pre-miRNA are processed in fact by a complex consisting of Dicer, TAR RNA-binding protein (TRBP) and protein activator of protein kinase PKR (PACT), facilitating loading of siRNA or miRNA duplex into Argonaute 2 (AGO2) and RNA-induced silencing complex (RISC). One of the two strands of each fragment, known as the guide strand, is then incorporated into the RISC. Argonaute proteins are the catalytic components of the RISC, AGO2 cleaving the passenger stand (Kim and Rossi 2007).

RNAi regulatory RNA molecules include synthetic siRNA, pol III transcribed small hairpin RNA (shRNA), or endogenous or artificial miRNA (Scherr and Eder 2007). These agents may be delivered by using either viral or nonviral vectors. Several animal models of human disease have been treated with such nucleic acids eliciting RNAi (Marsden 2006).

Advances in the nucleic acid chemistry, formulations and methods of delivery have created the possibility to develop effective RNAi-based therapeutics (Liu et al 2007). RNAi technologies offer higher efficiency to suppress the expression of specific genes, compared with traditional antisense approaches (Kurrek 2003; Wadhwa et al 2004; Bagasra and Prilliman 2004). Recently, improved targeting of miRNA with ASO was described (Davis et al 2006).

Although RNAi appears to be attractive for future therapeutics, controlling intracellular shRNA expression levels is very important, because oversaturation of cellular miRNA/shRNA pathways has been proven to be able to induce fatalities in adult mice (Grimm et al 2006).

RNAi triggered by siRNA

Over the last years researchers have put considerable effort into developing siRNA for therapeutic purposes (Karagiannis and El-Osta 2005). Introduction of siRNA into cells can cause

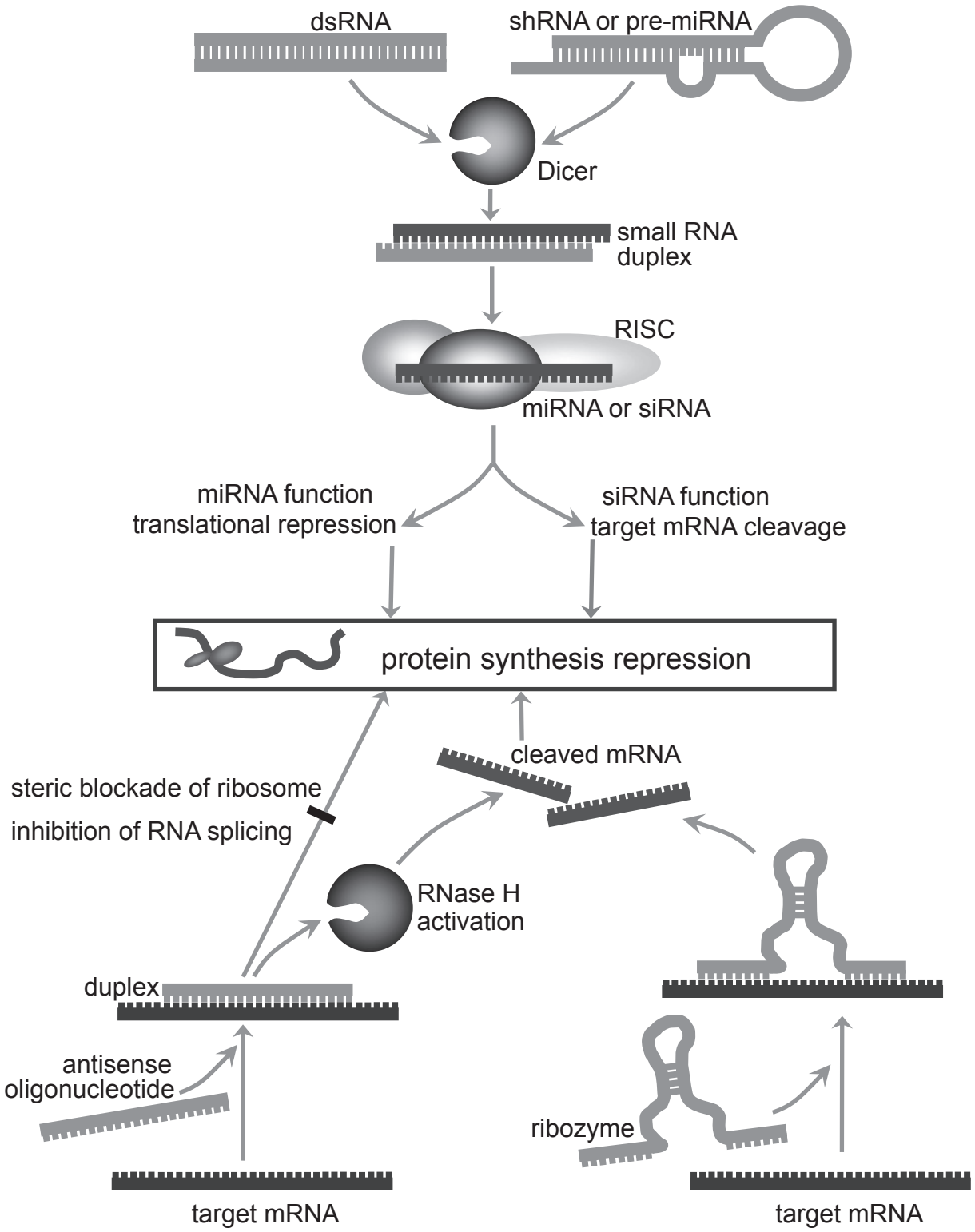

Figure 1 Schematic mechanisms of action of antisense therapeutic strategies.

sequence-specific gene silencing. Difficulties regarding the therapeutic application of siRNA are represented mainly by the delivery to the intracellular location in the target cells involved in the disease pathogenesis, and interaction with the RNAi machinery. In order to ameliorate these problems chemical modifications of siRNA, and viral and nonviral nucleic acid delivery systems have been developed (Oliveira et al 2006). A potential disadvantage of RNAi is represented

by the fact that the expression of RISC must be induced or may not be induced in all cell types, and may be saturated by multiple mRNA targets (Dreyfus et al 2004).

In a RNAi study, tyrosine-protein kinase Btk (Bruton's tyrosine kinase or Bruton agammaglobulinemia tyrosine kinase) was evaluated. Btk is a non-receptor tyrosine kinase encoded by the gene BTK (Xq21.3–q22) that plays a vital and complex modulatory role in many cellular processes. Increased IgE responses and exaggerated airway inflammation was reported in Btk-deficient mice (Kawakami et al 2006). Btk can transiently be depleted following transfection or microinjection of Btk siRNA duplexes, and the induction of histamine release mast cells was reduced by 20%–25% upon Btk down-regulation (Heinonen et al 2002).

Another molecular target for post-transcriptional silencing is represented by Ras guanyl nucleotide-releasing protein 4 (RasGRP4), a mast cell-restricted non-kinase intracellular signaling protein, with calcium- and diacylglycerol-binding motifs, required for the efficient expression of granule proteases in the mast cells. In an *in vitro* experiment, inhibition of RasGRP4 expression was obtained using a siRNA approach in a rat mast cell line, targeting RasGRP4 gene (19q13.1). siRNA-mediated inhibition of RasGRP4 expression in a similar cell line resulted in an inhibition of prostaglandin D_2 (PGD_2) synthase expression. Activated human mast cells release PGD_2, an eicosanoid mediator involved in the pathogenesis of allergic asthma. RasGRP4 regulates the expression of PGD_2 in human mast cell lines (Li et al 2003). Some experts consider that inhibition of histamine or PGD_2 release by mast cells, even mediated by RNAi, could have potential unwanted effects (Grzela et al 2004).

NF-κB is an important transcription factor that modulate airway inflammation in asthma due to its central role in the regulation of many immune and inflammatory processes, therefore it represents an interesting target for siRNA. The gene encoding transcription factor NF-κB p65 (nuclear factor NF-kappa-B p65 subunit) is RELA (11q13). siRNA directed to the p65 subunit of NF-κB (siRNA.p65) delivered by transfection in airway epithelial cells decreases levels of p65 mRNA and protein, and significantly inhibits the TNF-alpha-induced release of IL-6 and IL-8 (Platz et al 2005). These data indicate that RNAi has a potential use as a targeted therapy toward regulatory factors in the NF-κB pathway (Guo et al 2005).

Because ASO for the signal transducer and activator of transcription 6 (STAT-6) transfected in human lung epithelial or airway smooth muscle cells induce dose-dependent STAT-6 downregulation and prevent IL-13- and IL-4-dependent eotaxin release (Hill et al 1999; Peng et al 2004), STAT-6 is studied also as a target for siRNA. STAT6 gene is located on chromosome 12q13 and encodes a Th_2 differentiation transcription factor belonging to the family of cytokine-activated, tyrosine-phosphorilated transcription factors. STAT6 specific siRNA molecules induce the knock down of STAT6, blocking the release of eotaxin-3 in human epithelial cells stimulated with IL-4 and TNFalpha, while other pathways, such as the EGF stimulated release of IL-8, remain active (Rippmann et al 2005).

Recently it has been shown that Syk is widely expressed in airway epithelial cells and inhibition of Syk using siRNA down-regulated iNOS expression and reduced NO production. This effect appeared stimulated via $beta_1$-integrins. Inhibition of Syk down-regulated TNF-induced p38 and p44/42 MAPK phosphorylation and nuclear translocation of p65 NF-κB. Evaluation of the role of Syk in lung epithelial cells using RNAi may help in developing new therapeutic tools for asthma (Ulanova, Marcet-Palacios, et al 2006).

siRNA targeting key cytokines with important roles in allergic asthma are expected to be of benefit as RNAi immunotherapy. Other candidates considered as pharmacological targets for RNAi are TNF-alpha (Sorensen et al 2003) and the common beta-chain of the GM-CSF/IL-3/IL-5 receptor (Scherr et al 2003).

The safety profile of *in vivo* interventions using siRNA has to be evaluated in the future. These short RNA molecules have the potential to elicit immunological effects, including the induction of some proinflammatory cytokines and type I interferon. RNA-sensing immunoreceptors include members of the TLR family (TLR3, TLR7, TLR8) and cytosolic RNA-binding proteins (PKR, helicases RIG-I and Mda5). Type I interferon induction by synthetic siRNA requires TLR7 and is sequence dependent, similar to the detection of CpG motifs by TLR9 (Schlee et al 2006). It is possible to administer naked, synthetic siRNAs in animals, without inducing an interferon response (Heidel et al 2004). Experts must understand all aspects of such immune responses, including their implications for gene silencing technologies, if they want to minimize unwanted non-specific effects (Kawasaki et al 2004). Moreover, great attention is needed while evaluating potential severe side effects of long-term RNAi technologies before using in humans (Kurreck 2003).

Antagomirs

Antagomirs are a novel class of chemically engineered oligonucleotides (Krutzfeldt et al 2005), synthetic analogs of miRNA (Mattes et al 2007), that are powerful tools *in vivo* for

specific, efficient and long-lasting silencing of endogenous miRNA, an abundant class of non-coding or non-messenger RNA molecules believed to have important roles in many biological processes through regulation of gene expression. Parenteral administration of antagomirs induced in mice a significant reduction of corresponding miRNA levels in various tissues, including the lung. In antagomir-treated animals, gene expression and mRNA analysis revealed that regions of downregulated genes are depleted in miRNA recognition motifs (Krutzfeldt et al 2005).

Aberrant miRNA expression has been described in some human malignancies (Venturini et al 2007). Understanding the role of miRNA in asthma pathophysiology and the use of antagomirs may identify new potential biological targets for therapeutic intervention (Mattes et al 2007).

The identification of miRNA signatures was recently achieved in peripheral blood mononuclear cells isolated from a patient with asthma. Understanding the miRNA signature in susceptible individuals may potentially identify new drug targets. The future use of this new technology for the specific regulation of gene expression in respiratory diseases is awaited with enthusiasm (Mattes et al 2007).

Finally, several conclusions must be discussed. The deciphering of the human genome has revolutionized the strategy of nucleic-acid based technologies with sequences complementary to specific target genes. Promising antisense therapeutic strategies designed to specifically recognize and block the target mRNAs, have the potential to satisfy unmet needs in asthma treatment, acting at a more proximal level to a key patho-physiological molecular process, represented by abnormal expression of genes (Popescu 2005a).

Anti-mRNA approach in asthma can be achieved by using antisense oligonucleotides, ribozymes, and RNA interference (Sel et al 2006). siRNA is currently the fastest growing research field for target validation and therapeutic applications. Targeting mRNA rather than the protein itself is a more efficient approach to block a protein function, because multiple copies of a protein (approximately 5,000 copies) are produced by each mRNA molecule. There are several similar problems of effectiveness in all of these anti-mRNA methods: efficient delivery, enhanced stability, minimization or elimination of sequence- and substance-dependent side effects, and identification of sensitive sites in the target mRNAs (Scherer and Rossi 2003). Priorities and standards in antisense research is needed.

At present, there are no systemically administered nucleic acid-based therapeutics approved for human use. This reflects the difficulties in applying antisense agents, they are often nuclease sensitive and have difficulties in reaching their site of action. Nanotechnological strategies that improve the nucleic acids pharmacokinetics are recently suggested (Schiffelers et al 2007).

ASO are being developed for a wide array of therapeutic applications, while siRNA is now the gold standard method for analyzing gene function, and expectations for new target-specific drugs are high (Schlee et al 2006).

Although new biological treatments will be probably very expensive, their cost-effectiveness profile will be justified in some patients with severe refractory asthma (Bart 2007). After decades of difficulties, antisense interventions are relatively close to fulfilling their promise as therapeutics for clinical use. Further research is required in order to define an optimal strategy, because it is becoming ever clearer that antisense drugs can give rise to practical problems and their use in humans is more complicated than initially assumed. Despite all difficulties, antisense anti-asthma drugs are supposed to become a reality in the future (Grzela et al 2004).

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