Antisense- and RNA interference-based therapeutic strategies in allergy

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Abstract

Modern therapeutic methods for manipulation of gene expression in allergic diseases have been receiving increased attention in the emerging era of functional genomics. With the growing application of gene silencing technologies, pharmacological modulation of translation represents a great advance in molecular therapy for allergy. Several strategies for sequence-specific post-transcriptional inhibition of gene expression can be distinguished: antisense oligonucleotides (AS-ONs), ribozymes (RZs), DNA enzymes (DNAzymes), and RNA interference (RNAi) triggered by small interfering RNAs (siRNAs). Potential anti-mRNA drugs in asthma and other allergic disorders may be targeted to cell surface receptors (adenosine A₁ receptor, high-affinity receptor Fc-ε RI-α, cytokine receptors), adhesion molecules and ligands (ICAM-1, VLA-4), ion channels (calcium-dependent chloride channel-1), cytokines and related factors (IL-4, IL-5, IL-13, SCF, TNF-α, TGF-β1), intracellular signal transduction molecules, such as tyrosine-protein kinases (Syk, Lyn, Btk), serine/threonine-protein kinases (p38 α MAP kinase, Raf-1), non-kinase signaling proteins (RasGRP4), and transcription factors involved in Th2 differentiation and allergic inflammation (STAT-6, GATA-3, NF-κB). The challenge to scientists is to determine which of the candidate targets warrants investment of time and resources. New-generation respirable AS-ONs, external guide sequence ribozymes, and RNA interference-based therapies have the potential to satisfy unmet needs in allergy treatment, acting at a more proximal level to a key etiopathogenetic molecular process, represented by abnormal expression of genes. Moreover, antisense and siRNA technologies imply a more rational design of new drugs for allergy.

Keywords: allergy • antisense oligonucleotides • ribozymes • DNAzymes • RNA interference • small interfering RNAs

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Introduction

Advances in the understanding of the immunological mechanisms which initiate and mediate allergic reactions has led recently to a revised allergy nomenclature for global use [1]. Allergy is defined as a hypersensitivity reaction mediated by immunological mechanisms, which can be antibody- or cell-mediated. It is generally agreed that atopy is a personal or familial tendency to produce IgE antibodies in response to low doses of allergens, and, as a consequence, to develop typical symptoms of asthma, rhinoconjunctivitis or eczema. Inflammatory reactions, especially Th₂-driven ones and with eosinophilic involvement, are of fundamental importance in allergy. Allergic inflammation is characterized at molecular level by upregulated expression of genes encoding multiple inflammatory proteins, such as cytokines and chemokines, adhesion molecules, proinflammatory receptors, mediator-synthesizing enzymes, and dysregulated apoptosis in some inflammatory cells.

For modern molecular pharmacology, manipulating gene expression with new drug molecules is an important objective. In the emerging or expanding areas of therapeutic intervention in allergy, transcription and translation are the target events of great interest for modifying gene expression. While modulation of transcription usually influences the expression of multiple genes, new molecules acting on translation inhibit the expression of a single target gene. Post-transcriptional inhibition of gene expression at the mRNA level can be accomplished using antisense oligonucleotides (AS-ONs), ribozymes (RZs), DNA enzymes (DNAzymes), and small interfering RNAs (siRNAs). Antisense agents improved through chemical modifications, which inhibit in a sequence-specific manner the expression of target genes, may be used in allergy for functional genomics and target validation. Hybridization-based antisense and RNA interference (RNAi) technologies play also an important role in the development of allergy therapeutics. Because multiple copies of a protein (approximately 5,000 copies) are produced by each mRNA molecule, targeting mRNA rather than the protein itself is potentially a more efficient approach to block a protein function. There are several similar problems of effectiveness in all of these anti-mRNA methods: efficient delivery, enhanced stabil-

ity, minimization or elimination of sequence- and substance-dependent side effects, and identification of sensitive sites in the target mRNAs [2].

Current pharmacological research interests are represented mainly by new generation AS-ONs and siRNA molecules, which are investigated for their ability to modulate translation in asthma and allergic disorders [3]. For food allergy, another therapeutic option under investigation is genetic modification of foods. Antisense technology was used to reduce the allergenic protein content in transgenic rice seeds. This promising technique was also investigated for highly allergenic foods, such as peanut and soybean [4]. Genetically modified foods with few major allergenic epitopes have potentially a lower risk of inducing anaphylaxis.

Antisense and RNA interference targets in allergy

Allergy is caused by complex genetic and environmental interactions. Theoretically, any protein whose gene has been cloned can be targeted for regulation of gene expression. Although not every gene relevant to allergy, asthma or atopy is likely to represent a drug target for modifying expression, some antisense strategies have the potential to improve therapeutic benefits [5]. Anti-mRNA approaches depend on which particular genetic abnormality in allergy is being targeted. It is clear that research in this area should also give us more insights into the molecular mechanisms of allergic reactions.

Genome-wide linkage analysis in different populations have identified the locations of susceptibility genes for atopy and asthma, as well as associated phenotypes such as bronchial hyperresponsiveness and increased total serum IgE levels [6]. Some candidate genes have been recently identified using positional cloning approach. For example, ADAM33, a member of a gene family for proteins containing a disintegrin and metalloprotease domain, was recently reported as a major susceptibility gene, located on chromosome 20p13 [7]. Antisense deletion of ADAM33 in human altered airway fibroblasts inhibits the expression of contractile proteins, such as α -actin, induced by TGF- β and accelerates their apoptosis, explaining why this gene is involved in bronchial hyperresponsiveness

Table 1 Potential molecular targets for antisense and RNA interference-based therapy in allergy

Gene whose mRNA is the antisense target	Chromosomal location	Protein encoded
<i>Genes for cell surface receptors, adhesion molecules and ion channels</i>		
ADORA1	1q32.1	adenosine A ₁ receptor (AA1R)
FCER1A	1q23	Fc fragment of IgE, high affinity I, receptor α -subunit (Fc- ϵ RI- α)
IL4R (IL4RA)	16p11.2-p12.1	IL-4 receptor α chain (IL-4R- α , CD124)
IL5RA	3p26-p24	IL-5 receptor α chain (IL-5R- α , CD125)
CSF2RB	22q12.2-q13.1	GM-CSF/IL-3/IL-5 receptor common β -chain (CD131)
IL9R	2q31-q32	IL-9 receptor
EGFR	Xq28	epidermal growth factor receptor
ITGA4	7p12.3-p12.1	α -4 subunit of very late activating antigen-4 (VLA-4) receptor (CD49d)
ICAM1	19p13.3-p13.2	intercellular adhesion molecule-1 (ICAM-1, CD54)
CLCA1	1p31-p22	calcium-dependent chloride channel-1 (hCLCA1)
<i>Genes for cytokines and related factors</i>		
IL4	5q23-q31	interleukin 4 (IL-4)
IL5	5q23-q31	interleukin 5 (IL-5)
IL13	5q31	interleukin 13 (IL-13)
TNF (TNFA)	6p21.3	tumor necrosis factor- α (TNF- α)
KITLG	12q22	Kit ligand / stem cell factor (SCF)
TGFB1	19q13.1	transforming growth factor β -1 (TGF- β 1)
<i>Genes for non-receptor protein kinases, transcription factors and other intracellular signaling molecules</i>		
RAF1	3p25	RAF-1 proto-oncogene serine/threonine-protein kinase (Raf-1)
MAPK14	6p21.3-p21.2	mitogen-activated protein kinase 14 (p38 α MAP kinase)
LYN	8q13	tyrosine-protein kinase Lyn
SYK	9q22	tyrosine-protein kinase Syk (spleen tyrosine kinase)
BTK	Xq21.3-q22	tyrosine-protein kinase Btk (Bruton's tyrosine kinase)
RASGRP4	19q13.1	guanyl nucleotide-releasing protein 4 (RasGRP4)
RELA	11q13	transcription factor NF- κ B p65 (nuclear factor NF- κ B p65 subunit)
GATA3	10p15	trans-acting T-cell specific transcription factor GATA-3
STAT6	12q13	signal transducer and activator of transcription 6 (STAT-6)

and the progression of asthma [8]. The human genome project, as well as advances in the understanding of gene susceptibility and of key molecules in the pathophysiology of allergy, are revealing potential therapeutic antisense and RNAi targets (Table 1): cell surface receptors (G-protein coupled receptors, cytokine and chemokine recep-

tors), adhesion molecules, ion channels, cytokines, chemokines, growth factors, signal transduction receptor and non-receptor protein kinases or transcription factors involved in allergic inflammation or Th₂ differentiation, and other molecules [9, 10].

Although receptors and cytokines are frequently chosen targets for antisense interventions, various

classes of intracellular signal transduction molecules are also receiving increased attention, because dysregulated or aberrant signaling mechanisms are of significance in the pathogenesis of allergic diseases.

Recent advances in this sophisticated area of molecular research justify the presentation of studies that evaluate antisense- and RNA interference-based strategies blocking the inflammatory mediators or proinflammatory pathways in allergy. A great challenge to researchers and pharmaceutical industry is to determine which of the candidate targets warrants investment of time and resources.

Pharmacological modulation of translation in allergy

Antisense and siRNA technologies gained recently a greater attention in allergy and asthma research. Pharmacological approach of translation represents a great step in the promising age of molecular therapeutics because anti-mRNA agents are used for sequence-specific post-transcriptional inhibition of gene expression. Gene silencing nucleic acids, such as AS-ONs, RZs, DNazymes, and siRNAs, rely on hybridization to accessible sites within target mRNA for their action, and can be used to modify with high specificity the production of key proteins. Traditional drugs simply bind to these proteins or interfere with their activity, only temporarily preventing targeted protein function.

Standard allergy treatments, such as receptor agonists (β_2 -agonists, glucocorticosteroids), inverse agonists or antagonists (H_1 antihistamines, CysLT₁ leukotriene receptor antagonists) are used to reduce clinical manifestations, without eliminating the cause of allergy [11]. Because conventional therapies often fail to restore the immunological balance, and to fully control symptoms of allergy, sometimes being associated with adverse effects, there is a need for new treatment strategies.

A review of the studies that have been published on anti-mRNA approaches in asthma and other allergic disorders is important for scientists involved in the research area of molecular allergology. Experts working in the antisense and nucleic acid drug development field need critical evaluation of the methodologies used in these studies and

details about the specific features that relate to the application of antisense technology in allergy.

Cell culture systems have provided powerful means to evaluate antisense strategies *in vitro*, but it is unlikely to replace the need for transgenic animals or experimental models of asthma. Therapies using synthetic nucleic acids as anti-mRNA drugs can cause a number of different and often unexpected biological effects in cell cultures and live animals.

In studies with antisense oligonucleotides, the researchers must differentiate between the desired sequence-specific inhibition of the targeted mRNA from the undesired sequence-related and non-sequence-related effects, in order to choose and design appropriate therapeutic oligonucleotides [12]. Negative controls serve to rule out the possibility that the effects of AS-ONs 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 at least two different controls is recommended (sense controls, random controls, reverse controls, mismatch controls). Sense controls are oligonucleotides having a sequence complementary to that of the AS-ON (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 AS-ON bases in a randomized manner, while mismatch controls are obtained by the deliberate introduction of one or more mismatches into the antisense sequence [12]. In siRNA studies it is important to design and test two to four siRNA sequences *per* gene, to purify *in vitro* transcribed siRNA and avoid RNases. The usage of positive and negative controls, optimized transfection reagents and protocols is essential for a successful experiment.

Different assay systems are used to evaluate antisense-mediated effects, measuring the target mRNA levels (Northern blots and RT-PCR) and measurement of protein levels (Western blots, ELISA, immunoprecipitation). Biological assays, binding assays, cell proliferation, morphological and functional analysis can also be used.

Although many species of animals can serve as models for allergic disease in humans, there is no animal model that exactly reproduces the pathology of human disease. Animal models of allergic asth-

ma, especially those involving rodents and nonhuman primates, are necessary for the development of antisense therapies [13]. Mouse, guinea pig and rat models of asthma are still used in many studies, but disadvantages must be considered, including difficulties related to the techniques for measuring pulmonary function, and species differences in expression or responses to cytokines and other mediators [14]. Large animal models of allergic asthma have been developed in rabbits, dogs, cats, sheep, rhesus and cynomolgus monkeys. Nonhuman primate models that allow many of the experimental manipulation performed in rodents, are much closer to chronic asthma in terms of immunology and histopathology, and are most suitable because antisense therapy with nucleic acids that specifically target the human immune system is less likely to be active in animals less genetically similar to man [14]. Several antisense technologies are used to modulate translation in allergy.

Antisense oligonucleotides (AS-ONs)

AS-ONs are usually single-stranded synthetic nucleic acid polymers, consisting of 15–25 nucleotides, used in research since the late seventies. Their sequence-specific effects (antisense effects) are due to the hybridization with the target mRNA in a sequence-dependent complementary manner, binding being performed exclusively *via* hydrogen bonds. Most AS-ONs are designed to activate RNase H, which cleaves the RNA moiety of a DNA-RNA heteroduplex, leading to the degradation of the targeted mRNA. AS-ONs that do not induce RNase H cleavage can be used to inhibit translation by steric blockade of the ribosome, with translational arrest. In addition, AS-ONs can be used to correct aberrant splicing [15].

First generation AS-ONs are phosphorothioate (PS) oligodeoxynucleotides with one of the non-bridging oxygen atoms in the phosphodiester bond substituted by sulfur, in order to enhance nuclease resistance. PS-ONs containing unmethylated deoxycytosine-deoxyguanosine (CpG) motifs are able to trigger unwanted immunostimulatory responses. A major disadvantage of PS-ONs is their binding to certain proteins, possibly causing cellular toxicity. The reason for this nonspecific interaction is not completely understood.

Second generation AS-ONs contain nucleotides with alkyl modifications at the 2' position of the ribose. 2'-*O*-methoxyethyl-oligonucleotides (2'MOE-ON) are important members with increased target binding affinity and resistance to degradation. Because they do not recruit RNase H, the antisense effect can be due to the steric blockade of translation.

Other compounds with alternative structures are third generation AS-ONs: peptide nucleic acids (PNAs), N3'-P5' phosphoroamidates (NPs), 2'-deoxy-2'-fluoro- β -d-arabino nucleic acids (FANAs), locked nucleic acids (LNAs), morpholino oligonucleotides (MF), cyclohexene nucleic acids (CeNA), and tricyclo-DNA (tcDNA) oligonucleotides [15].

In the allergy treatment, AS-ONs can be used to downregulate the gene expression for many targets: cell membrane receptors (G-protein coupled receptors, cytokine receptors), adhesion molecules and ligands, ion channels (Table 2), cytokines and related factors (Table 3), signaling non-receptor protein kinases (tyrosine kinases, such as Lyn, Syk, Btk, and serine/threonine kinases, such as Raf, MAP kinases) and transcription factors, such as Cys4 zinc finger (GATA-3) or β -scaffold factors with minor groove contacts (p65, STAT-6) (Table 4), etc.

Respirable antisense oligonucleotides (RASONS)

are effective at doses that are smaller than for other routes of administration, because are delivered as aerosols directly to the lungs, thus minimizing the potential for non-antisense systemic side effects and toxicity [16]. *In vivo* absorption, tissue distribution, metabolism and excretion, efficacy and safety studies of respirable AS-ONs suggest that these antisense agents can be safely delivered to target respiratory tissues in low, but efficacious doses [17, 18].

Aerosolized adenosine A₁ receptor AS-ON (EPI-2010) is a respirable PS-ON targeting A₁ receptor discordantly overexpressed in the allergic lung [17]. Preclinical studies in rabbits and primates revealed that EPI-2010 attenuated adenosine- and allergen-induced bronchial obstruction (Table 2). The effect of EPI-2010 on the inflammatory markers, such as bronchoalveolar lavage (BAL) cellularity and lung immunohistopathology were not reported, but the ability to block airway hyperresponsiveness (AHR) to inhaled adenosine persisted several days, giving it the potential to be a once-*per*-week treatment for asthma at the time of phase I clinical trials. EPI-2010 is safe and well tolerated, but Phase IIa clini-

Table 2 AS-ONs targeting cell surface receptors, adhesion molecules and channels in allergy

AS-ONs target [reference]	AS-ONs -route of administration, -experimental model	AS-ONs effects (comments)
Adenosine A ₁ receptor [24]	AA1R AS-ON (PS-ON) -inhalation (EPI-2010), -house-dust mite-sensitized rabbit and <i>Ascaris</i> -sensitized primate asthma models, humans with asthma	-reduce adenosine A ₁ receptors number in bronchial smooth muscle, -improve significantly allergen-induced airway obstruction and AHR, -only modest efficacy in humans with mild asthma (phase I/IIa clinical trials)
Fc-ε RI-α [25]	Fc-ε RI-α AS-ON (PS-ON, FITC labeled) -in cell cultures/topically, -rat PMC, IgE-dependent PCA	-reduce Fc-ε RI-α expression in PMC, -dose-dependent PCA reaction inhibition, -inhibit histamine and TNF-α release from PMC (anti-DNP IgE-activated/mediated)
IL-5R-α [26]	IL-5R-α AS-ON (2'MOE-ON) -in cell cultures/systemic (intravenous), -BCL-1 cell cultures, fetal liver- and bone marrow-derived cells/IL-5 and ragweed-induced allergic peritonitis murine model	- <i>in vitro</i> decreases mRNA and protein expression, selectively inhibit IL-5 induced eosinopoiesis from murine fetal liver and bone marrow cells, - <i>in vivo</i> reduce blood and bone marrow eosinophilia in the IL-5-induced peritonitis model, -but also inhibit the development of blood and tissue eosinophilia in the allergic peritonitis model
GM-CSF/IL-3/IL-5 R-β [27]	GM-CSF/IL-3/IL-5 R-β AS-ON (PS-ON) -in cell cultures, -human purified eosinophils	-decrease β chain of IL-3/IL-5/GM-CSF receptor mRNA expression, -inhibit eosinophil survival
GM-CSF/IL-3/IL-5 R-β [28]	GM-CSF/IL-3/IL-5 R-β AS-ON (AS143 PS-ON) -intratracheal, -rat allergic asthma model	-reduce lung β chain of IL-3/IL-5/GM-CSF mRNA and protein expression, -inhibit antigen-induced lung eosinophilia and AHR to LTD ₄
CD49d subunit of VLA-4 [29]	CD49d AS-ON (2'MOE-ON) (ISIS 107248/ATL-1102) -inhalation (low dose), -OVA-challenged mouse lung	- <i>in vitro</i> reduce CD49d mRNA, - <i>in vivo</i> inhibit allergen-induced eosinophil lung infiltration, mucus production, and AHR to methacholine
ICAM-1 [30]	ICAM-1 AS-ON (PS-ON) (alicaforsen) -systemic (formulation with atelocollagen as carrier), -allergic dermatitis in mice	-suppress the inflammatory response in mice sensitized by dinitrofluorobenzene, evaluated by ear thickness, histopathology and immunochemistry, -atelocollagen enhances antisense activity
gob-5 (hCLCA1 counterpart) [31]	gob-5 AS-ON (adenoviral vector) -intratracheal -mouse model of asthma	-inhibit gob-5 expression, -prevent goblet cell hyperplasia, -suppress AHR and mucus overproduction

AS-ON = antisense oligonucleotide; PS = phosphorothioate; 2'MOE = 2'-O-methoxyethyl; OVA = ovalbumin;
 FITC = fluorescein isothiocyanate; DNP = dinitrophenyl; PMC = peritoneal mast cells; BCL-1 = B-cell lymphoma 1;
 PCA = passive cutaneous anaphylaxis; AHR = airway hyperresponsiveness.

Table 3 AS-ONs targeting cytokines and related factors in allergy

AS-ONs target [reference]	AS-ONs - route of administration, - experimental model	AS-ONs effects (comments)
IL-4 [32, 33, 34]	IL-4 AS-ON (PS-ON) - in cell cultures, - rat spleen cells; murine B cells cocultured with Th ₂ clone	- inhibit IL-4 production from a Th ₂ clone, - inhibit polyclonal IgE, but not antigen-specific IgE production <i>in vitro</i>
IL-4 [35]	IL-4 AS-ON treated CD4 ⁺ cells from OVA-sensitized rats, transferred to naive rats	-decrease IL-4 expression, -inhibit the adoptively transferred late phase allergic response
IL-4 [36]	IL-4 AS-ON (PS-ON) -ex vivo incubation (FITC labeled) -nasal mucosa biopsy specimens (ragweed allergic rhinitis)	-decrease allergen-induced IL-4 mRNA, -inhibitory effects on allergen-induced epsilon germline transcript RNA expression and mucosa eotaxin 1 immunoreactivity
IL-13 [37]	IL-13 AS-ON (PS-ON) -in cell cultures, -tonsil human B lymphocytes cultured with anti-CD40 monoclonal antibodies and rIL-4	-block B-cell IL-13 production, -inhibit IgE production <i>in vitro</i>
SCF [38]	SCF AS-ON (PS-ON) -intranasal (FITC-labeled), -OVA-sensitized mice with asthma	-inhibit SCF expression in interstitial lung cells, -reduce IL-4 concentration in BAL fluid, -decrease eosinophil and mast cell counts in lung interstitial tissue and/or BAL fluid, as effective as corticosteroid treatment
TGF-β1 [39]	TGF-β1 AS-ON (PS-ON) -in cell cultures/topically, -rat PMC/IgE-dependent PCA	-reduce TGF-β1 expression in PMC, -dose-dependent PCA reaction inhibition, -inhibit histamine and TNF-α release from PMC (anti-DNP IgE-activated/mediated)
IL-5 [40]	IL-5 AS-ON (2'MOE-ON) -intravenous (ISIS 20391), -murine OVA-induced asthma and allergic peritonitis model	-inhibit IL-5 mRNA and IL-5 protein expression, -reduce antigen-induced lung eosinophilia, lack of immunostimulation, -inhibit the late phase AHR
TNF-α [41]	TNF-α AS-ON (2'MOE-ON) (ISIS 104838) -inhalation, -mouse model of chronic asthma	-reduce TNF-α protein in BAL fluid, -inhibit recruitment of eosinophils and neutrophils to the airways, decreases lung inflammation, -reduce mucus production and AHR

AS-ON = antisense oligonucleotide; PS = phosphorothioate; 2'MOE = 2'-O-methoxyethyl; OVA = ovalbumin;
FITC = fluorescein isothiocyanate; DNP = dinitrophenyl; PMC = peritoneal mast cells; BAL = bronchoalveolar lavage;
PCA = passive cutaneous anaphylaxis; AHR = airway hyperresponsiveness.

Table 4 AS-ONs targeting non-receptor protein kinases and transcription factors in allergy

AS-ONs target [reference]	AS-ONs -route of administration, -experimental model	AS-ONs effects (comments)
Lyn [42, 43]	Lyn AS-ON (PS-ON) -in cell cultures, -OVA-sensitized murine bone marrow cells	-block Lyn expression in eosinophils, -prevent IL-5 receptor cascade activation and inhibit eosinophil differentiation from stem cells, attenuate antiapoptotic IL-5 effect on eosinophils (similarly to Raf-1 AS-ONs, which in contrast inhibit also ECP secretion)
Syk [21, 22, 44]	Syk AS-ON (PS-ON/liposome complexes) -inhalation, - <i>Nippostrongylus</i> larvae / OVA-induced asthma in rats	-reduce Syk mRNA and protein expression in inflammatory cells, -depress up-regulation of some adhesion molecules (ICAM-1, α_4 and β_2 integrins), -suppress antigen-mediated trachea contraction, -inhibit nitric oxide and TNF release from alveolar macrophages, -inhibit antigen-induced pulmonary inflammatory cell infiltrate, decreasing both eosinophilia and neutrophilia
p38 α MAP kinase [45]	p38α MAPK AS-ON (2'MOE-ON, ISIS 101757) -aerosol for inhalation or nose-only exposure, -OVA-sensitized mice with asthma	-inhibit p38 α mRNA and protein expression in BAL fluid cells and peribronchial lymph node cells, -reduces mucus hypersecretion, -suppress Th ₂ cytokine production (reduce IL-4, IL-5 and IL-13 levels in BAL fluid, Th ₁ cytokine levels being unaffected), -inhibit airway eosinophilia and AHR
p65 subunit of NF-κB [46]	p65 AS-ON (PS-ON) -systemic (intravenous), -OVA-sensitized mice with asthma	-suppresses the allergen-specific IgE response -reduce eosinophil number, TNF- α and Th ₂ cytokines, IL-4 and IL-5, in BAL fluid, -inhibit eosinophilic infiltration and AHR
GATA-3 [20]	GATA-3 AS-ON (PS-ON) -intranasal (FITC-labeled), -OVA-sensitized murine model of asthma	-reduce intracellular GATA-3 lung expression due to DNA uptake in lung cells, -suppress Th ₂ cytokine production, -inhibit eosinophilic infiltration and AHR
STAT-6 [47]	STAT-6 AS-ON (PS-ON) -transfection of cell lines, -human lung epithelial cells	-dose-dependent STAT-6 mRNA and protein downregulation
STAT-6 [48]	STAT-6 AS-ON (PS-ON) -transfection of cell lines, -human airway smooth muscle cells	-selective downregulation of STAT6 protein, -prevent IL-13- and IL-4-dependent, but not IL-1 β -dependent, eotaxin release

AS-ON = antisense oligonucleotide; PS = phosphorothioate; 2'MOE = 2'-O-methoxyethyl; OVA = ovalbumin; FITC = fluorescein isothiocyanate; AHR = airway hyperresponsiveness; BAL = bronchoalveolar lavage; ECP = eosinophil cationic protein.

cal trials revealed modest indications of efficacy in patients with mild asthma [3]. In my opinion, the treatment with adenosine A₁ receptor AS-ON failed

to confirm the anticipated effectiveness because the pathophysiology of human asthma is complex, involving many immunological abnormalities, not

only a single receptor with proinflammatory and bronchoconstrictive actions.

EPI-30051, a respirable AS-ON targeting the T cell specific transcription factor GATA-3 [19], has the potential advantage of simultaneously down-regulation of various Th₂ cytokines expression (Table 4), rather than suppressing a single mediator [20]. Aerosolized Syk AS-ON may also provide a new therapeutic approach in allergic asthma (Table 4), because by depressing the expression of a protein tyrosine kinase critical for FcR-mediated signaling and activation of various immune cells, this PS-ON inhibits the release of mediators from alveolar macrophages, the up-regulation of important adhesion molecules, and pulmonary inflammation [21, 22].

Second-generation respirable AS-ONs targeted to IL-4 receptor α chain, p38 α mitogen-activated protein (MAP) kinase, CD49d subunit of VLA-4, and TNF- α are potential treatment modalities for lung allergic inflammation, recently receiving more attention from medical scientists.

The IL-4 receptor α 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 Th₂ differentiation, and by blocking its production is inhibited the activity of IL-4 and IL-13, that regulate allergic inflammation, mucus overproduction, and AHR. The IL-4 receptor α chain is currently studied as a target for new inhaled second generation AS-ONs (ISIS 369645).

VLA-4 (very late antigen-4) is the $\alpha_4\beta_1$ integrin (CD49d/CD29) localized on many inflammatory cells that participates in cell adhesion, trafficking and activation, through binding to VCAM-1 and fibronectin [23]. Small molecule VLA-4 inhibitors have demonstrated inhibition of AHR and lung inflammation in animal asthma models. The VLA-4 α subunit (CD49d) may be a target for second generation AS-ONs (ISIS 107248), also known as ATL-1102 (Table 2).

TNF- α (tumor necrosis factor- α) is implicated as a causative factor for chronic allergic bronchial inflammation, being a cytokine involved in many effects associated with airway epithelium (alteration of permeability, upregulation of ICAM-1 expression, stimulation of IL-6, IL-8 and GM-CSF production) and other relevant effects in the lungs. TNF- α AS-ONs (ISIS 104838) are studied in the treatment of rheumatoid arthritis and psoriasis, and also in persistent asthma (Table 3).

p38 MAP kinase is a non-receptor proline-directed serine/threonine kinase with a pivotal role in the activation of inflammatory cells. Small molecule inhibitors of this signal transduction kinase decrease proinflammatory cytokine production, reduce chemotaxis, and enhance eosinophil apoptosis, however toxicity provides an obstacle in the development of such agents acting on p38 MAP kinase. p38 α MAP kinase AS-ONs (ISIS 101757) are more selective than traditional drugs, and administration as aerosols further reduces the risk of adverse effects (Table 4).

Ribozymes (RZs) and deoxyribozymes (DNA enzymes or DNazymes)

RZs are synthetically engineered, catalytically active RNA molecules, capable of cleaving target mRNA (RNA enzymes), with high sequence specificity. Since the discovery of the first group I intron RZs in the early eighties, new classes of RZs, including hammerhead and hairpin RZs, have been developed [49]. Hammerhead RZs, the most advanced in therapeutic applications, present 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. AUC and GUC trinucleotides are processed most efficiently. Chemical modification of RZs are made for stabilization and nuclease resistance. Stabilization of RZs is more difficult than protection of AS-ONs [15]. Zinzymes cleave YGH target sequences (where Y is C or U, and H is A, C or U). Hairpin RZs usually cleave after BNGUC (where B can be any nucleotide except adenosine).

The 10–23 DNazyme is capable of cleaving mRNA in a highly sequence-specific manner at sites that contain purine-pyrimidine junctions [50]. An advantage of DNazymes over RZs is that they are less expensive and more stable than RZs [51].

Allergy represents a clinical area suitable for RZ use. Many key peptides/proteins involved in the immunopathogenesis are identified for disorders such as allergic rhinoconjunctivitis and asthma. RZs can be used to cleave the mRNA encoding specific peptide/protein targets [52].

Hammerhead and hairpin RZs targeting conserved sequences within IL-5, NF- κ B and ICAM-1 mRNA have been already designed. Pharmacokinetic studies

in mice revealed that intratracheal RZs administration increase exposure in lungs [53].

External guide sequence (EGS)

An external guide sequence (EGS) ribozyme targeting the human IL-4 receptor α mRNA was recently studied, having in mind that no adverse effects related to the loss of IL-4 function were noted at pulmonary level or systemically, in human subjects. Catalytic RNA termed EGS and RNA interference use small RNA molecules to inactivate a target mRNA. A sequence EGS1 was designed based on the similarity to the tRNA cleavage consensus sequence and location in exposed regions of the human IL-4 receptor α mRNA. EGS1 directs efficient RNase P-mediated cleavage of mRNA for human IL-4R mRNA. Inhaled small nuclease-resistant EGSs, such as EGS1, introduced into pulmonary tissues using endogenous lung surfactants, as described with conventional respirable AS-ONs, represent a potential new therapeutic approach in asthma [54]. Blockade of the IL-4 receptor α chain is able to inhibit the pathogenic effects of both IL-4 and IL-13 using a single antisense agent.

Inhaled EGS ribozymes could have advantages over conventional antisense DNA oligonucleotides due to their different mechanisms of action, involving the ability to act as a co-catalyst for the ubiquitous RNase P, and because of the potential for a single EGS to recycle. Reduced off-site cleavage by EGS is an advantage, not only over conventional antisense DNA oligonucleotides, but also relative to RNA interference [54]. There are experimental data that suggest that the effect of EGSs is apparent more rapidly compared with siRNA [55].

RNA interference (RNAi) triggered by small interfering RNAs (siRNAs)

RNA interference and the described external guide sequences are important methods that use RNA to suppress gene expression in human cells. RNA interference (RNAi), or RNA silencing, is triggered by siRNAs, which can be generated in several ways. Long double-stranded RNA (dsRNAs) molecules are processed into 21–23 nucleotide small interfering RNAs (siRNAs) by the Dicer enzyme (a RNase III-like dsRNA-specific endonu-

lease, acting as a dimer that cleaves both strands of dsRNAs leaving two-nucleotide, 3' overhanging ends). Alternatives are represented by exogenous siRNAs that can be provided as synthesized oligonucleotides or expressed from plasmid or viral vectors.

siRNA molecules are incorporated into the RNA-induced silencing complex (RISC), that includes helicase, RecA, and exo- and endonucleases, as well as other proteins. Following its assembly, the RISC guides the RNA degradation machinery to the target mRNAs and cleaves it in a sequence-specific, siRNA-dependent manner. siRNA molecules for RNAi applications offer higher efficiency to suppress the expression of specific genes, compared with traditional antisense approaches [15, 56, 57].

A potential disadvantage of RNAi *versus* EGS is represented by the fact that the expression of RISC must be induced, may not be induced in all cell types, and may be saturated by multiple mRNA targets, while RNase P is present permanently, in significant amounts, in all cell type [54].

The first report of a presumable RNAi benefit in the molecular pharmacology of allergic diseases [58] revealed that Bruton's tyrosine kinase (Btk) protein, a non-receptor tyrosine kinase that plays a vital and complex modulatory role in many cellular processes, can transiently be depleted following transfection or microinjection of Btk siRNA duplexes, and that the induction of histamine release in RBL-2H3 mast cells was reduced by 20–25% upon Btk down-regulation.

Another *in vitro* evaluation of siRNA in allergy experimental pharmacology was recently performed [59]. siRNA-mediated inhibition of RasGRP4 expression in the same type of rat mast cell line resulted in an inhibition of prostaglandin D₂ (PGD₂) synthase expression. RasGRP4 (Ras guanyl nucleotide-releasing protein 4) is 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. Activated human mast cells release PGD₂, an eicosanoid mediator involved in the pathogenesis of systemic mastocytosis, allergic asthma, and rhinitic responses. RasGRP4 regulates the expression of PGD₂ in human mast cell lines [59]. Some experts consider that inhibition of histamine or PGD₂ release by

mast cells, even mediated by RNAi, could have potential unwanted effects, similar to that observed after antihistamine or anti-arachidonic acid metabolites treatment [11]. But great care has to be taken while ruling out severe side effects of long-term RNAi applications [15].

siRNA can also be used to regulate the activity of NF- κ B in cells involved in allergic inflammation. Application of siRNA directed to the p65 subunit of NF- κ B (siRNA.p65) by transfection in airway epithelial cells causes decreased levels of p65 mRNA and protein, and significantly inhibits the TNF- α -induced release of IL-6 and IL-8 [60]. RNAi represents a powerful tool to better define the function of regulatory factors in the NF- κ B pathway, and has a potential use as a targeted therapy [61].

Other candidates considered as pharmacological targets for RNAi are TNF- α [62] and the common beta-chain of the GM-CSF/IL-3/IL-5 receptor [63]. siRNAs, with systemic or topical delivery, targeting key cytokines that play a critical role in allergy immunopathogenesis, are expected to be of benefit as RNA interference immunotherapy.

Recently, the safety profile of siRNAs has been reevaluated. Both siRNA and hybridization-based antisense agents have been shown to exert non-target-related biological effects, including immune stimulation, possible by acting as agonists of some Toll-like receptors (TLR), belonging to a family of pathogen-associated, molecular pattern recognition receptors [64]. TLR9 specifically recognizes unmethylated CpG motifs in synthetic double-stranded (ds) or single-stranded (ss) oligonucleotides [65]. The majority of PS-ONs contain CpG dinucleotides, and their position and the sequences flanking play critical role in determining immunostimulatory activity [64]. Non-CpG-containing 2'MOE-ONs have a lower proinflammatory risk, independent of TLR9 [66].

siRNAs have the potential to elicit immune responses *via* interactions with TLR3, and trigger interferon responses like long dsRNA and its analogs [67]. TLR3 was described as an external dsRNA pattern recognition receptor found in many cell types, and ssRNA has been shown to interact with TLR7 and TLR8. It is possible to administer naked, synthetic siRNAs in animals, without inducing an interferon response [67]. Experts must understand all aspects of immune responses to dsRNA, and its implications for gene silencing

technologies, in order to minimize unwanted non-specific effects [68].

Finally, it must be noted that DNA methylation was recently identified as another RNA silencing mechanism [68].

Conclusions

New methods used for selective post-transcriptional modulation of gene expression in allergic diseases have potential advantages over conventional antiallergic drugs, in the emerging era of functional genomics. Receptors, cytokines, adhesion and signaling molecules provide interesting antisense or RNAi targets, that offer modern opportunities to interfere with regulatory pathways of allergic inflammation.

Antisense and RNA interference-based therapeutical strategies designed to specifically recognize and block the target mRNAs, have the potential to satisfy unmet needs in allergy treatment, acting at a more proximal level to a key etiopathogenetic molecular process, represented by abnormal expression of genes. Moreover, antisense and siRNA technologies imply a more rational and a possible less expensive design of new drugs for allergy.

Antisense oligonucleotides and RNA interference technology are recent approaches in allergy treatment supposed to become a reality in the future [11]. Currently, pharmacological manipulation of translation is at an early stage. But already from the evidence available at this moment, I can underline that second and third generation AS-ONs, EGS ribozymes, and siRNAs will be probably the preferred anti-mRNA strategies in order to modulate the most important molecular targets in allergy.

After a review of what has been accomplished using antisense oligonucleotides, ribozymes, and RNA interference-based methods, many challenges still remain. Prioritizing the vast information in need of investigation is required in order to obtain more rapidly new drug molecules for allergy, characterized by optimal efficacy and selectivity. Guidance to experts on how to apply the antisense technology to treating allergy is also of great interest. Safety profile must be careful eval-

uated in the progress from cell culture studies and *in vivo* animal models to clinical trials.

Further studies will be needed in the future to evaluate antisense therapies in areas not only regarding efficacy and safety, but also comparison or concomitant administration with traditional drugs, the altering of the natural course of the allergic disease or the cost-effectiveness ratio. All these aspects will provide usefulness for the future clinical applications of anti-mRNA or gene silencing therapies.

Traditional anti-gene agents, antisense oligonucleotides and ribozymes, and the new promising anti-gene strategy, RNA interference, which disrupt the expression of disease-related genes, could potentially be of utility not only in the treatment of allergy, but also in the therapeutics of many other diseases, including cancer [69, 70–77].

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