Antisense molecules: A new class of drugs

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An improved understanding of disease pathogenesis leads to identification of novel therapeutic targets. From a pharmacologic point of view, these can be addressed by small chemical compounds, so-called biologicals (eg, mAbs and recombinant proteins), or by a rather new class of molecule based on the antisense concept. Recently, a new wave of clinical studies exploring antisense strategies is evolving. In addition to cancer, they include predominantly trials on infectious and noninfectious diseases, such as chronic inflammatory and metabolic conditions. This article, based on a systematic PubMed literature search, highlights recent developments in this emerging field. (J Allergy Clin Immunol 2016;137:1334-46.)

Key words: *Clinical trial, antisense, small interfering RNA, DNAzyme, therapy*

Abbreviations used

ALS:	Amylotrophic lateral sclerosis
AMD:	Age-related macular degeneration
CMV:	Cytomegalovirus
CNV:	Choroidal neovascularization
CVD:	Cardiovascular disease
DMD:	Duchenne muscular dystrophy
FDA:	US Food and Drug Administration
HCV:	Hepatitis C virus
IRS-1:	Insulin receptor substrate 1
LDL-C:	Low-density lipoprotein cholesterol
MOE:	2′-O-methoxyethyl
ODN:	Antisense oligodeoxyribonucleotide
PCSK9:	Proprotein convertase subtilisin/kexin type 9
PT:	Phosphorothioate
siRNA:	Small interfering RNA
TLR:	Toll-like receptor
VEGF:	Vascular endothelial growth factor

In recent years, we are observing the advent of novel therapeutic strategies in many disease areas. These strategies are primarily based on advancement in our understanding of disease mechanisms, resulting in identification of novel therapeutic targets. This includes chronic inflammatory diseases, such as allergies, asthma, chronic inflammatory bowel disease, rheumatoid arthritis, and other autoimmune disorders. Further examples are metabolic and cardiovascular diseases (CVDs), eye diseases, some neurologic disorders, and also infectious diseases, particularly in the field of viral infections. These developments go hand in hand with the ambitious strategy to provide novel individualized and personalized therapeutic approaches. From a pharmacologic point of view, the development of novel therapeutic strategies faces several major challenges: many targets are located intracellularly, thus requiring membrane passage of the drug molecules, and further challenges include the stability, systemic availability, and half-life of the compound and safety issues.

To address these challenges, several major avenues are being explored, including small (synthetic) molecules, mAbs, and antisense strategies. The antisense technology is primarily used to directly interfere with the machinery of the production of target proteins through inactivation of (m)RNA encoding for the target protein. This field has received great attention since the report of successful early-stage clinical studies, which have been performed in several disease areas, including allergy and asthma.

A variety of reviews appeared over the last years, focusing on the comparative description of different antisense approaches and the development of novel modifications of antisense molecules to overcome initial obstacles for their use as medicines.[1-7] However, thus far, there is no comprehensive overview available summarizing the current status of the application of such compounds in clinical trials with some exception to the field of cancer.[8,9]

The aim of this review is to provide an update on this fascinating and rapidly developing field receiving increasing attention in the scientific and medical community. We will introduce the concept of the major antisense approaches, followed by an update on the clinical application of antisense molecules in the area of noncancer disorders.

ANTISENSE STRATEGIES

The term antisense molecules comprises several classes of oligonucleotide molecules that contain sequence complementarity to target RNA molecules, such as mRNA, viral RNA, or other RNA species, and that inhibit the function of their target RNA after sequence-specific binding. This goes back to its first

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Supported by the PreDicta EC FP7 Collaborative Project (260895), Universities Giessen and Marburg Lung Centre (UGMLC), the German Centre for Lung Research (DZL; 82DZL00502), and the German Research Council (DFG; SFB1021, project C04).
Disclosure of potential conflict of interest: D. P. Potaczek, S. D. Unger, and H. Renz have received grants from PreDicta EC FP7 Collaborative Project (260895), Universities Giessen and Marburg Lung Centre (UGMLC), the German Centre for Lung Research (DZL; 82DZL00502), and the German Research Council (DFG; SFB1021, project C04). H. Garn has received grants from PreDicta EC FP7 Collaborative Project (260895), Universities Giessen and Marburg Lung Centre (UGMLC), the German Centre for Lung Research (DZL; 82DZL00502), and the German Research Council (DFG; SFB1021, project C04) and has consultant arrangements with and has received stock/stock options from Sterna Biologicals GmbH & Co. KG.
Received for publication August 11, 2015; revised November 24, 2015; accepted for publication December 15, 2015.
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The CrossMark symbol notifies online readers when updates have been made to the article such as errata or minor corrections
0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2015.12.1344

TABLE I. General features of the main antisense molecule families

	Antisense DNA (ODN)	siRNA	DNAzyme	RNAzyme
Oligo type and structure	Linear ssDNA	Linear dsRNA	ssDNA with 2 binding domains surrounding a central catalytic domain	Complex RNA with single- and double-strand sections
Size	12-25 bases	21-25 base pairs	30-35 bases	• >30 bases (hammerhead RNAzymes) • >50 bases (hairpin RNAzymes)
Inherent enzymatic activity	No	No	Yes	Yes
Recruited enzymatic activity	RNAses H, L, or P	RISC	No*	No
Modifications	Multiple backbone modifications established, such as PT, 2-O-methyl, 2-O-methoxyethyl, locked nucleic acids	Several modifications, preferentially of terminal nucleotide overhangs established, such as 3′-inverted thymidine	• 3′-Inverted thymidine to enhance stability • Modifications in binding arms possible	Often unmodified (especially when expressed *in vivo*), several modifications possible
Advantages	• Easy to produce, easy to modify • Good cell penetration, especially for modified short antisense DNAs	• Can be generated intracellularly from larger precursors by enzyme Dicer • Degradation of whole mRNA • Multiple turnover	• Easy to produce • Multiple potential cleavage sites in target RNAs • Good *in vivo* cell penetration • No significant off-target effects shown thus far • Multiple turnover	• Multiple potential cleavage sites in target RNAs • Can be expressed from coding DNA sequences *in vivo*
Disadvantages	• Off-target effects • Potential protein-binding (aptamer) activities • Activity dependent on intracellular enzymes	• Off-target effects • More difficult to produce • Limited cell penetration potential • Activity dependent on intracellular enzymes	• Empiric selection process • Activity depends on intracellular Mg^{2+} levels	• Large size and complex structure • Empiric selection process • Difficult to produce • Limited *in vivo* stability

dsRNA, Double-stranded RNA; *RISC*, RNA-induced silencing complex; *ssDNA*, single-stranded DNA.
*Might also involve RNAses H, L, or P.

description in 1978, when the antiviral activity of antisense DNA molecules against the Rous sarcoma virus was shown.[10,11] In the meantime, at least 4 major classes of antisense molecules have been described: antisense oligodeoxyribonucleotide (ODN), ie, single-stranded DNA molecules, small interfering RNA (siRNA) molecules, ribozymes, and DNAzymes (Table I).[1]

The by far most frequently used application of therapeutic antisense molecules is to reduce expression levels of proteins that have been associated with central mechanisms of disease development. In this regard antisense molecules bind to the mRNAs that have been generated by transcription from the genetic information in the nucleus. These mRNAs serve as coding molecules for the formation of respective proteins in a process called translation. Binding of antisense molecules can effectively inhibit this translational process because mRNAs associated with antisense molecules can no longer serve as matrices. Thus the expression level of intact mRNAs and, subsequently, their coded proteins can be reduced by effective antisense action (Fig 1). However, antisense strategies can also be developed to interfere with other functional aspects of RNA molecules, such as blocking of splicing, interference with appropriate folding, competing with protein binding to RNA sequences, antagonizing microRNA activities, and inhibiting RNA-mediated telomerase activity.[2] Initially, it was thought that antisense molecules exert their activity through steric blocking of the target RNA with subsequent inhibition of RNA functionality. More detailed analyses of the mode of action of antisense molecules revealed that many of these structures involve enzymatic activities to fully exert their function (Table I). In the case of RNAzyme and DNAzyme, this activity is inherent in the antisense molecule itself, as the names indicate, and thus these molecule families comprise enzymatically active oligonucleotides. ODNs can recruit intracellular enzymes, such as ribonucleases (RNAses) H, L, or P, which cleave RNA on binding of the ODN molecule. siRNAs represent short RNA duplex molecules 21 to 25 bases long that involve the generation of the so-called RNA-induced silencing complex (RISC), which exerts ribonuclease activity with subsequent cleavage of the RNA target.

A variety of modifications were developed and applied to the different kinds of antisense molecules, mainly to increase their stability (Table I). Modifications should also enhance the bioavailability of these molecules, especially for therapeutic use, and improve binding to and inactivation of target RNAs. One of the first and still most widely applied modifications is the introduction of phosphorothioate (PT)–containing nucleotides to form a PT backbone in which oxygen atoms in the bridging structures are replaced by sulfur atoms (first-generation antisense drugs). This modification significantly increases the stability of antisense molecules in biological systems; however, it has been also associated with a variety of unwanted side effects.[12] Other backbone modifications include phosphoramidate, methylphosphonate, phosphorodiamidate morpholino, or peptide nucleic acids.

More recent modifications (second- and third-generation antisense drugs) introduce changes into the heterocycle or (deoxy)ribose sugar to increase the stacking interaction with

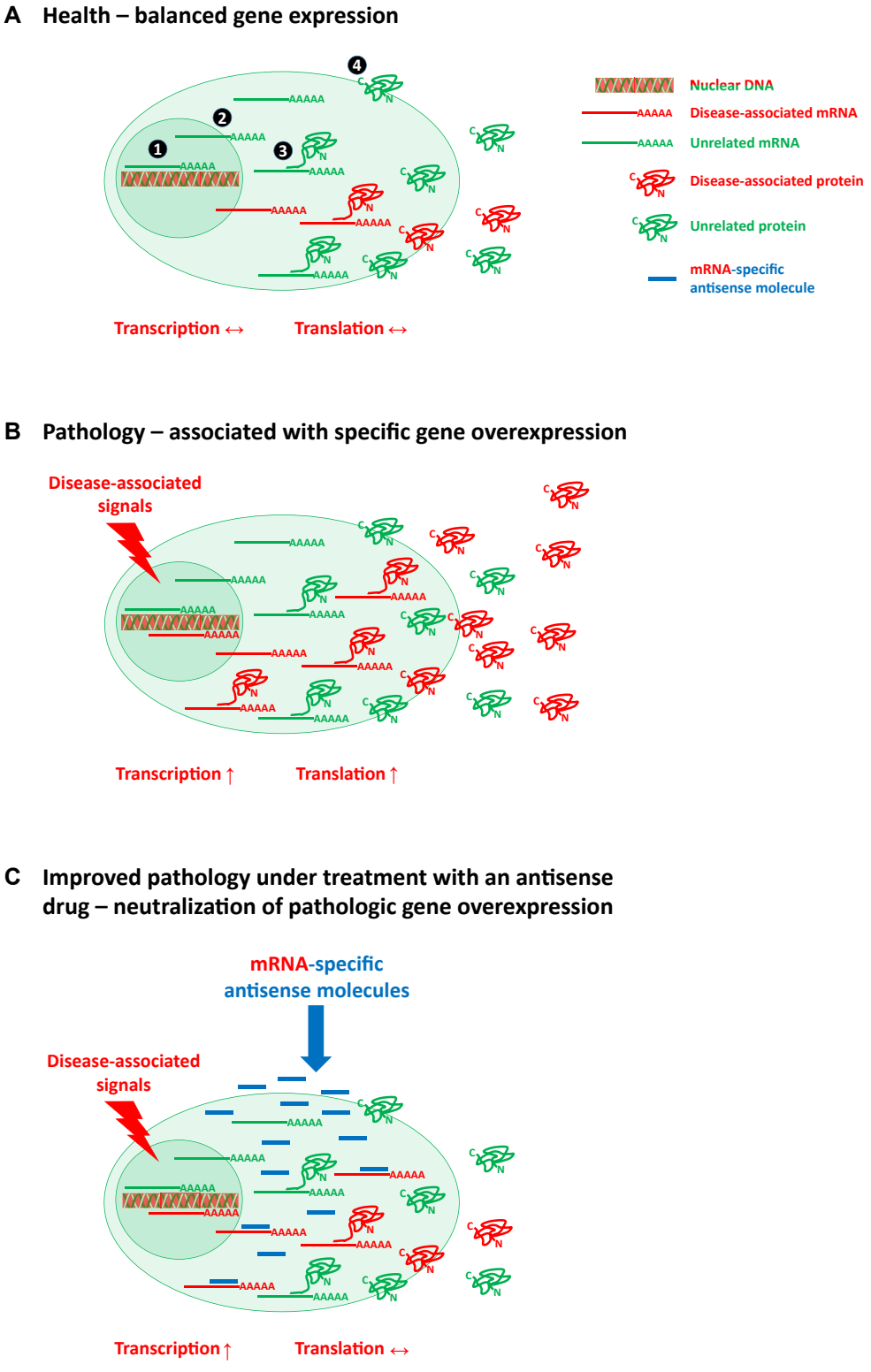

FIG 1. Mode of action of antisense molecules as inhibitors of disease-associated protein expression. **A,** Under healthy conditions, cells permanently generate a variety of proteins needed for their own homeostasis and interaction with their environment in a tightly controlled process. Gene-specific mRNAs are transcribed from the respective DNA regions in the nucleus *(1)*, and mRNAs are transferred to the cytoplasm *(2)*. At the ribosomes, mRNA information is translated into the corresponding protein sequence *(3)*. After appropriate processing, proteins are transported to their final location, which might be intracellular, membrane bound, or secreted to the extracellular space *(4)*. **B,** Following disease-associated signals, the steady state of cells becomes disturbed, and transcription/translation of a variety of (disease-associated) genes/proteins can be significantly increased. This leads to a dysregulated cellular response, which drives the pathological process. **C,** Highly gene-specific antisense molecules can bind and subsequently block or even cleave targeted disease-associated mRNA molecules. This interaction inhibits translation into the respective protein and rebalances cellular activities toward normal conditions.

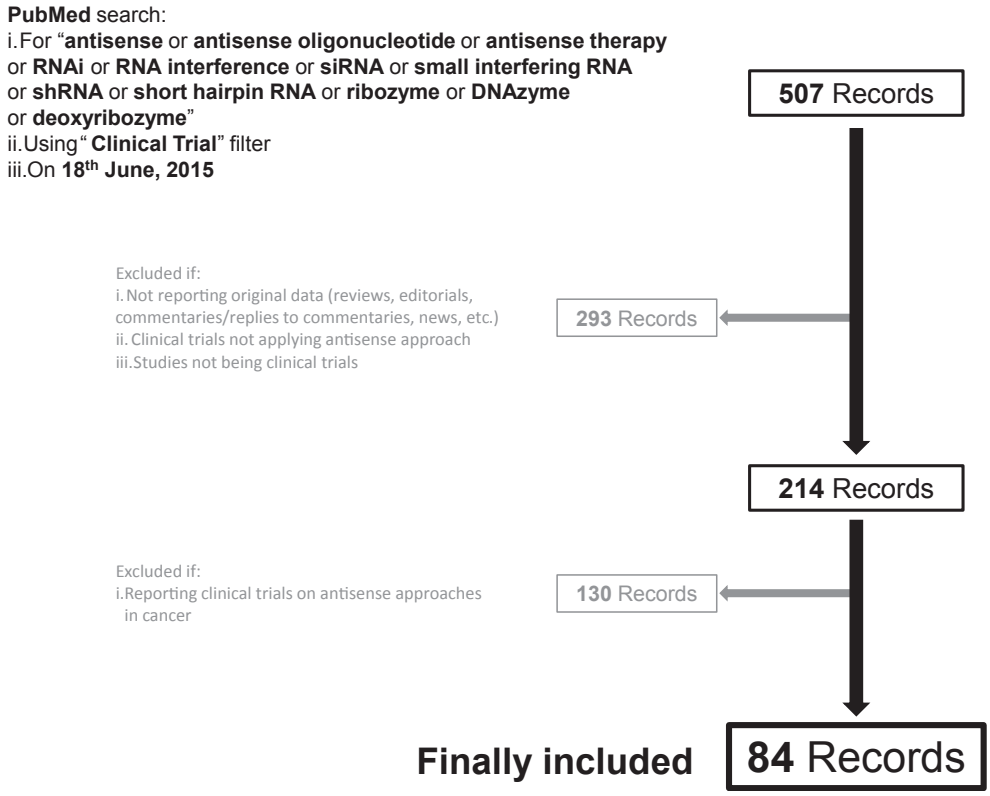

FIG 2. Strategy of the systematic literature search and its results.

the target molecule and nuclease resistance. Among the latter are 2′-O-methyl, 2′-O-methoxyethyl (MOE), and 2′-O-fluoro modifications. Most recently, so-called bicyclic and locked nucleic acids were developed that show significantly increased hybridization properties to target structures and high stability in biological fluids.[3] However, most modifications are also associated with an altered toxicological profile requiring intense investigation of unwanted side effects for the respective antisense-based drug compound.

SYSTEMATIC SEARCH

To cover the area of interest, we conducted a systematic literature search. In brief, on June 18, 2015, the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed) was searched by using the input "antisense OR antisense oligonucleotide OR antisense therapy OR RNAi OR RNA interference OR siRNA OR small interfering RNA OR shRNA OR short hairpin RNA OR ribozyme OR DNAzyme OR deoxyribozyme," restricting the results with the "Clinical Trial" filter, which yielded a total of 507 hits. These were subsequently subjected to abstract-based and, if necessary, full text–based screening to exclude further articles (1) not reporting original data (eg, reviews, editorials, commentaries/replies to commentaries, and news), (2) clinical trials not applying an antisense approach, and (3) studies that were not clinical trials, which resulted in elimination of 293 articles. From the remaining 214 articles, a further 130 papers describing the results of clinical trials on antisense-based treatment conducted in different types of cancer were also excluded (Fig 2). Cancer is an important area of antisense drug application; however, involvement of studies in this field would have expanded the article to an unmanageable size. The final 84 articles were then studied in detail and subgrouped based on the clinical area, the pathophysiologic character of addressed molecules, and/or the target of an antisense approach. The designs and major results of the trials reported in those 84 articles are briefly summarized in Table E1 in this article's Online Repository at www.jacionline.org. Subjectively selected studies are also discussed in the text and presented in Tables II-VI.[13-65]

INFLAMMATORY DISORDERS

Inflammatory diseases are multifactorial and complex disorders that require therapy with rather unspecific anti-inflammatory compounds (eg, corticosteroids and calcineurin inhibitors). For many reasons, there is need for more specific therapeutic approaches interfering with central molecules in crucial pathomechanistic pathways. These might represent molecules involved in the recruitment, activation, or both of inflammatory cells, such as molecules from cytokine/chemokine networks, receptors and signaling molecules, cell adhesion molecules, and transcription factors controlling the expression of sets of genes responsible for the development of certain disease conditions. Thus a variety of antisense approaches were developed over the last about 20 years that were intended to treat chronic inflammatory disease conditions in different organ systems (Table I and see Table E1).

One of the first antisense molecules applied in clinical studies was alicaforsen (ISIS 2302), a 20mer PT DNA molecule directed against the adhesion molecule intercellular adhesion molecule 1. Because intercellular adhesion molecule 1 is important for the recruitment of inflammatory cells, such as neutrophils, to the site

TABLE II. Overall output of selected clinical trials on antisense drugs related to inflammatory disorders

Target	Antisense approach (drug name)	Disease (potentially) targeted	General outcomes	References
GATA-3	DNAzyme (SB010)	Asthma	Attenuation of late- and early-phase asthmatic responses in allergen provocation study after drug inhalation Reduced systemic IL-5 levels Good safety profile, low systemic exposure	Homburg et al, 2015[23]; Krug et al, 2015[24]
CCR3 and cytokine common β-chain	Combination of 2 ODNs (TPI ASM8)	Asthma	Reduction in allergen-induced early- and late-phase asthmatic responses Reduction in sputum eosinophil counts Good safety profile	Gauvreau et al, 2008[20]; Gauvreau et al, 2011[21]; Imaoka et al, 2011[22]
ICAM-1	ODN (alicaforsen, ISIS 2302)	Crohn disease	Some trends to higher frequency of patients with remission, especially in prespecified patients Trends to reduced glucocorticoid consumption Postinfusion drug reactions, otherwise well tolerated	Yacyshyn et al, 1998[13]; Yacyshyn et al, 2002[14]; Yacyshyn et al, 2005[15]
ICAM-1	ODN (alicaforsen, ISIS 2302)	Ulcerative colitis	Different outcomes of studies regarding change of disease activity index Improvement in duration of standard-care treatment responses Favorable safety profile	van Deventer et al, 2004[16]; Miner et al, 2006[18]; van Deventer et al, 2006[17]
SMAD7	ODN (GED0301)	Crohn disease	Higher numbers of patients with clinical remission Reduced plasma levels of proinflammatory mediators Safe and well tolerated	Monteleone et al, 2012[55]; Zorzi et al, 2012[56]; Monteleone et al, 2015[19]

ICAM-1, Intercellular adhesion molecule 1.

TABLE III. Overall output of selected clinical trials on antisense drugs related to viral infections

Target	Antisense approach (drug name)	Disease (potentially) targeted	General outcomes	References
miR-122	ODN (miravirsen, SPC3649)	HCV infection	Long-lasting reductions in plasma HCV levels in patients with chronic infection accompanied by a good safety profile	Janssen et al, 2013[28]
Major IE2 protein-encoding CMV mRNA	ODN (fomivirsen, Vitravene)*	CMV retinitis accompanying AIDS	Effectiveness in patients with newly diagnosed, relapsed, or persistent retinitis based on clinically assessed parameters Well tolerated	Vitravene Study Group, 2002[29]; Vitravene Study Group, 2002[30]; Vitravene Study Group, 2002[31]
RSV nucleocapsid N protein mRNA	siRNA (ALN-RSV01)	RSV infection	Lower rate of infection in experimentally inoculated healthy subjects and reduced incidence of bronchiolitis obliterans in lung transplant recipients Good safety profile	DeVincenzo et al, 2008[32]; DeVincenzo et al, 2010[33]; Zamora et al, 2011[34]
Overlapping vpr and tat genes of HIV-1 RNA	Gammaretroviral vector expressing hammerhead ribozyme delivered within transduced cells (RRz2, OZ1)	HIV-1 infection	Long-term engraftment of transduced cells Reduction in some measures of viral load in HIV-1–infected patients Procedure feasible and well tolerated	Amado et al, 2004[25]; MacPherson et al, 2005[26]; Mitsuyasu et al, 2009[27]

CMV, Cytomegalovirus; *IE2*, immediate-early region 2; *RSV*, respiratory syncytial virus.
*Approved by the FDA for local treatment of CMV retinitis in patients with AIDS who are intolerant or have a contraindication to other treatments for CMV retinitis or who were insufficiently responsive to previous treatments for CMV retinitis.[80]

TABLE IV. Overall output of selected clinical trials on antisense drugs related to metabolic diseases

Target	Antisense approach (drug name)	Disease (potentially) targeted	General outcomes	References
Apolipoprotein B (−100 [ApoB −100])	ODN (mipomersen, ISIS 301012, Kynamro)*	Hypercholesterolemia, increased cardiovascular risk	Reduction in LDL-C, ApoB, and Lp(a) levels in different forms of hypercholesterolemia Injection-site reactions and flu-like symptoms among most typical side effects Clinically relevant (≥3× upper limit of normal) ALT increase in some patients not accompanied by significant changes in bilirubin levels and synthetic liver function; transient hepatic steatosis in few cases No drug-drug interactions with simvastatin, ezetimibe, or warfarin	Kastelein et al, 2006[36]; Yu et al, 2009[35]; Akdim et al, 2010[57]; Akdim et al, 2010[58]; Visser et al, 2010[59]; Raal et al, 2010[37]; Akdim et al, 2011[60]; McGowan et al, 2012[61]; Visser et al, 2012[62]; Stein et al, 2012[63] Thomas et al, 2013[64]; Li et al, 2014[65]
PCSK9	siRNA (ALN-PCS)	Hypercholesterolemia	Reduction in plasma PCSK9 and LDL-C levels in subjects with near-optimal or higher LDL-C levels Good safety profile	Fitzgerald et al, 2014[39]
Apolipoprotein C-III (ApoC-III)	ODN (ISIS 308401)	Hypertriglyceridemia	Reduction in plasma ApoC-III and triglyceride levels in healthy volunteers No major safety concerns	Graham et al, 2013[40]

ALT, Alanine transaminase; *Lp(a)*, lipoprotein(a).
*Approved by the FDA as complementary treatment of patients with homozygous familial hypercholesterolemia.[38]

of inflammation, this molecule is overexpressed in a variety of inflammatory disease conditions, including chronic inflammatory intestinal disorders. In fact, after phase I safety studies[66] demonstrating a favorable safety profile, alicaforsen was first applied in patients with Crohn disease in a variety of trials. Although systemic application through intravenous infusion did not significantly improve the rate of remissions, several studies described positive effects on reduction of glucocorticoid consumption,[13,67] Crohn disease activity index, and/or inflammatory bowel disease score.[14] Later studies demonstrated a significant correlation with remission in a subpopulation of patients carrying single nucleotide polymorphisms in the gene encoding TNF-α[15]; however, no significant clinical response was observed in relation to previous use of anti–TNF-α antibody therapy.[68] Rectal formulation of the same antisense drug through an enema was applied for the treatment of ulcerative colitis and chronic pouchitis in several studies, resulting in dose-dependent improvement of disease activity index[16,69] and prolonged durability of treatment effects compared with mesalazine standard therapy.[17,18] Systemic application of alicaforsen in patients with rheumatoid arthritis[70] and renal allograft rejection[71] did not result in significant treatment effects.

Regarding Crohn disease, a new approach was recently developed based on an antisense DNA molecule against the transcription factor SMAD7. SMAD7 acts as an inhibitor of the immunosuppressive cytokine TGF-β1 signaling cascade and has been shown to be upregulated in the colons of patients with Crohn disease. Interestingly, the SMAD7-specific antisense drug mongersen is applied orally by using a polymer-coated tablet. Treatment with this compound was demonstrated to be safe and well tolerated, and a recently finished phase IIa study revealed significantly higher numbers of patients with clinical remission at day 15 and maintenance of remission for at least 2 weeks in the 2 higher-dose groups (40 or 160 mg/d, respectively) compared with placebo.[19]

A combination of 2 antisense DNA molecules called TPI ASM8 was developed and investigated in a series of 3 clinical studies for the treatment of allergic asthma. Although one component of this drug is directed against the chemokine receptor CCR3, which is crucially involved in the attraction of eosinophils, mast cells, and basophils to the site of inflammation, the other antisense molecule corresponds to the mRNA of the cytokine receptor common β-chain, which is an essential part of IL-3, IL-5, and GM-CSF receptors, all of which are involved in the generation and recruitment of eosinophils. The inhaled application of TPI ASM8 showed a favorable safety profile.[20] Furthermore, in allergen provocation studies a significant reduction in asthmatic early- and late-phase responses was observed and paralleled by an improvement in airway hyperresponsiveness and reduction in sputum eosinophil numbers and target gene expression.[21,22]

Most recently, a DNAzyme-based drug was successfully applied in a clinical study for the first time. SB010 represents a DNAzyme directed against GATA-3, the master transcription factor of T_H2-driven immune responses. GATA-3 is mainly expressed in T_H2 cells themselves, where it controls expression of the T_H2 subtype–specific cytokines IL-4, IL-5, and IL-13 and is thus crucially involved in the differentiation and activation of this specific T-cell subtype. Moreover, GATA-3 is also expressed in effector cells of allergic inflammatory responses, such as eosinophils, mast cells, basophils, and (bronchial) epithelial cells, and thus represents an interesting target for an antisense-based treatment strategy for T_H2-driven allergic inflammatory disease conditions, such as allergic asthma. In fact, inhaled application

TABLE V. Overall output of selected clinical trials on antisense drugs related to ocular diseases

Target	Antisense approach (drug name)	Disease (potentially) targeted	General outcomes	References
RTP801	siRNA (PF-04523655)	Neovascular AMD, diabetic macular edema	Improvement in visual acuity vs comparator alone in subjects with neovascular AMD or diabetic macular edema Generally safe and well tolerated	Nguyen et al, 2012[41]; Nguyen et al, 2012[42]; Nguyen et al, 2012[43]
Insulin receptor substrate-1 (IRS1)	ODN (aganirsen, GS-101)	Progressive corneal neovascularization secondary to keratitis or keratouveitis	Beneficiary effects on corneal neovascularization, such as inhibition, reduction/regression, and/or stabilization Generally safe and well tolerated	Kain et al, 2009[44]; Cursiefen et al, 2009[45]; Cursiefen et al, 2014[46]
β_2-Adrenergic receptor (ADRB2)	siRNA (SYL040012)	Glaucoma	Reduction of IOP in healthy subjects with higher baseline IOP values Safe and well tolerated	Moreno-Montañés et al, 2014[47]
Gap junction protein, alpha 1 (connexin 43)	ODN (Cx43AsODN)	Persistent severe ocular burns	Rapid reduction of inflammation in subjects with persistent severe chemical and/or thermal ocular burns unresponsive to established therapy Complete and stable corneal re-epithelialization	Ormonde et al, 2012[48]

IOP, Intraocular pressure.

TABLE VI. Overall output of selected clinical trials on antisense drugs related to diseases not covered by the previous sections ("miscellaneous")

Target	Antisense approach (drug name)	Disease (potentially) targeted	General outcomes	References
Dystrophin gene mutant mRNA with premature stop codon	RNA (PRO051, drisapersen, GSK2402968)	DMD	Reintroduction of open reading frame with production of quasinormal protein and performance improvement in patients Good safety profile	Van Deutekom et al, 2007[50]; Goemans et al, 2011[51]; Voit et al, 2014[52]
Integrin, α4 (CD49D, α subunit of VLA-4 receptor)	ODN (ATL1102)	MS	Reduced disease activity in patients with relapsing-remitting MS Fairly good safety profile	Limmroth et al, 2014[53]
Coagulation factor XI	ODN (ISIS 416858)	VTE	Lower incidence of VTE in patients undergoing knee arthroplasty compared with standard protection Good safety profile	Büller et al, 2015[54]
Transthyretin	siRNA (ALN-TTR01 and ALN-TTR02*)	Transthyretin amyloidosis	Rapid and durable decrease in mutant and wild-type transthyretin levels in patients and healthy subjects Fairly good safety profile	Coelho et al, 2013[49]

MS, Multiple sclerosis; *VLA-4*, very late antigen 4; *VTE*, venous thromboembolism.
*Representing 2 formulations of the same siRNA.

of SB010 was demonstrated in a series of 3 phase 1 studies in healthy and asthmatic subjects to be safe and well tolerated.[23] A subsequent phase IIa study provided evidence for significant improvement of early- and late-phase asthmatic responses to an allergen challenge after treatment with the GATA-3–specific DNAzyme-based drug, as demonstrated by significantly attenuated area under the curve for FEV_1. These clinical improvements were associated with a marked attenuation of allergen-induced sputum eosinophilia and lower sputum tryptase and serum IL-5 levels.[24] The latter studies are promising examples of the potential of antisense-based drugs as locally applied treatment options with high therapeutic efficacy and limited (systemic) side effects.

VIRAL INFECTIONS

In general, viral infections are addressed either symptomatically (eg, common colds) or indirectly (eg, stimulation of antiviral response by interferons). Until quite recently, drugs specifically inhibiting viral replication had not been broadly available. This situation started to change in the last 2 to 3 decades, with substantial progress made in the development of more specific therapeutics against hepatitis viruses, influenza, and especially HIV. Nevertheless, there is still much room for improvement, which could be filled by an application of antisense strategies. By their nature, antisense drugs can also offer much better (antiviral) specificity.

In comparison with other antisense strategies described in this review, those against viruses are characterized by substantially higher methodological complexity and heterogeneity, resulting mostly from virus-to-virus differences in the structure of genomic material (single- or double-stranded DNA or RNA, positive- or negative-sense single-stranded RNA, and reverse transcriptase) and thus expression and/or replication biology. On the other hand, additional RNA types, eg, negative sense RNA synthesized during replication of the genome of single-stranded positive sense RNA viruses, transiently appearing within the lifecycle of some viral pathogens increase the number of potential targets for antisense (anti-RNA) therapy.

The most interesting, although complex, approach has been recently tested in the case of HIV with the aim of not only controlling the infection but potentially curing the host as well (Table III and see Table E1).[25-27,72,73] In principal, $CD4^+$ T cells or $CD34^+$ progenitor cells were harvested, *ex vivo* transduced and re-expanded, and then reinfused. Vectors used for transduction encoded antisense molecules, such as ribozyme, short hairpin RNA, and/or long antisense RNA, the sustained expression of which was present even a few years after engraftment of the transduced cells. In all studies the procedure was safe, without any adverse effects different from those typical for autologous cell transplantation. Finally, the results of the 2 studies analyzing direct and/or indirect antiviral efficacy of this approach were at least partly promising.[27,73] Although further improvements and optimizations are probably required, the strategy based on transduction of autologous cells with anti-HIV molecule–expressing vectors seems to create a chance of developing a therapy bringing long-term or even curative effects. Achieving these goals would be possibly much more difficult with current antiviral drugs or simpler HIV-targeting antisense approaches.[74,75]

The strategy involving a plasmid expressing antiviral antisense molecules has also been used in the case of hepatitis B virus against which a vector encoding 4 different short hairpin RNAs (NUC B1000) has been developed. Although shown to be safe after intravenous infusion, it has not been yet tested for its antiviral efficacy.[76] Also, hepatitis C virus (HCV) became a target of antisense approaches. Although the PT ODN directly binding to HCV genomic RNA (ISIS 14803) did not prove to be strikingly effective,[77,78] a very promising result has been reported for a locked nucleic acid PT ODN targeting microRNA-122 (miravirsen, SPC3649), a human-encoded molecule required for an effective HCV propagation (Table III and see Table E1).[28,79] It is possible that such an indirect variant of antisense strategy could be a good solution in case of some viruses, especially those in which genomic material cannot be effectively approached because of its strong folding.

In addition to those applied systemically, topically administered antiviral antisense drugs, such as those against cytomegalovirus (CMV) retinitis (fomivirsen [Vitravene]; Novartis, Basel, Switzerland),[29-31] or respiratory syncytial virus (RSV) infection/bronchiolitis (ALN-RSV01),[32-34] have been clinically studied, with very favorable results (Table III and see Table E1). Intravitreally injected fomivirsen, a PT ODN targeting CMV mRNA encoding major immediate-early region 2 proteins, was shown to be effective and well-tolerated in patients with AIDS and newly diagnosed, relapsed, or persistent CMV retinitis in phase III trials. In 1998 and as a first antisense molecule, it received US Food and Drug Administration (FDA) approval for the local treatment of CMV retinitis in patients with AIDS who are intolerant or have a contraindication to other treatments for CMV retinitis or who were insufficiently responsive to previous treatments for CMV retinitis.[80] Successful in terms of both safety and efficiency, data obtained for ALN-RSV01, a multiply modified siRNA against RSV mRNA encoding the nucleocapsid N protein delivered as a either nasal spray or nebulization, suggest in turn that also other viruses infecting the upper and/or lower respiratory tract, eg, influenza or human rhinoviruses, could eventually be targeted with antisense approaches.

METABOLIC DISEASES

Atherosclerosis is a thickening of the arterial wall resulting from an accumulation of white blood cells, especially macrophages, absorbing oxidized low-density lipoprotein cholesterol (LDL-C) to form so-called "fatty streaks," a crucial step in the development of atherosclerotic plaque. These changes have 2 major consequences: (1) narrowing of the arterial lumen results in blood flow limitation and chronic ischemia in the supplied organs and (2) atherosclerotic plaques tend to become unstable and vulnerable, which can lead to plaque erosion and rupture, with subsequent thrombosis suddenly closing the artery, followed potentially by acute infarction. These 2 processes lead to the chronic or acute symptoms of CVD, respectively, with the clinical picture depending on the arterial bed.[81-84] Thus, not surprisingly, dyslipidemia, especially hypercholesterolemia, is one of the major risk factors of atherosclerotic changes leading to the development of CVD.[85,86] Apolipoprotein B-100 (ApoB-100) is an essential structural and receptor-binding component of all atherogenic lipoproteins, including LDL and its metabolic precursors. Hence specific targeting of ApoB-100 by means of antisense strategies should make it possible to reduce LDL-C levels and therefore the risk of CVD.[87,88] Indeed, an MOE-modified PT ODN against ApoB-100 (ISIS 301012, mipomersen [Kynamro]; Genzyme, Cambridge, Mass) has been developed and widely tested in clinical trials (Table IV and see Table E1). After initial studies showed neither relevant drug-drug interactions nor inhibition of major CYP enzymes[35] and provided acceptable safety results,[36] several subsequent efficacy trials have been conducted in populations with different forms of dyslipidemia, varying in terms of severity, pathogenesis (several studies on heterozygous or homozygous familial hypercholesterolemia), accompanying disorders or susceptibility to them (coronary artery disease and increased CVD risk), and/or response to standard lipid-decreasing treatment (stable therapy or maximally tolerated doses). Together, they have shown a significant (and dose-dependent) reduction in circulating LDL-C, ApoB, and, where investigated, lipoprotein (a) levels in subjects treated with subcutaneous mipomersen.[36,37,57-64] Of note, those trials included not only phase II but also phase III clinical studies, in which collectively almost 400 subjects were randomized 2:1 to weekly receive 200 mg of mipomersen or placebo for 26 weeks.[37,61,63,64,89] Treatment with mipomersen is associated with some side effects, including increases in liver enzyme levels (although without significant changes in bilirubin levels and synthetic liver function), (transient) hepatic steatosis, and flu-like symptoms. The mechanistic reasons for these adverse effects have not been fully elucidated. Established lipid-decreasing therapy with statins makes it possible to control cholesterol levels in the majority of patients. In some subjects

at high CVD risk with marked hypercholesterolemia, such as those with familial hypercholesterolemia, however, standard medications are not capable of adequately reducing LDL-C levels despite maximally tolerated doses, thus making a space for mipomersen and possibly other antisense approach–based hypolipidemic drugs.[85,87,88] Successful clinical trials of mipomersen have already resulted in its approval by the FDA as complementary treatment of patients with homozygous familial hypercholesterolemia.[38]

Proprotein convertase subtilisin/kexin type 9 (PCSK9), the enzyme whose binding to LDL receptors leads to their degradation, represents another potential antisense target involved in LDL-C biology. Recently, a multiply modified, lipid nanoparticle–encapsulated siRNA against PCSK9 (ALN-PCS) has demonstrated a good safety profile and efficiency in reducing PSCK9 plasma protein and LDL-C levels on intravenous infusion in healthy subjects with near-optimal or higher LDL-C levels.[39] Apolipoprotein C-III (ApoC-III), an independent risk factor and key regulator of plasma triglyceride levels, has been addressed by an antisense strategy as well. Subcutaneously injected MOE-modified PT ODN (ISIS 308401) has not only been proved safe but also demonstrated to reduce plasma ApoC-III and triglyceride concentrations in healthy subjects.[40] The results of the latter 2 studies are very promising, but we need to wait for phase II trials data to determine whether the substances they tested will repeat the success of mipomersen.

OCULAR DISEASES

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness in the developed world. Choroidal neovascularization (CNV), the hallmark of the neovascular form of AMD, is responsible for most cases of severe vision loss resulting from AMD.[90-92] After the discovery of vascular endothelial growth factor (VEGF) as a key stimulatory factor in angiogenesis, it became a promising target for novel treatment modalities of CNV caused by neovascular AMD. Indeed, anti-VEGF therapies have demonstrated a potential to inhibit disease progression and improve vison.[90-93] Antisense strategies have been also directed at CNV caused by AMD by mean of direct targeting of the VEGF pathway, specifically VEGF receptor 1. In patients with this condition, intravitreally injected anti–VEGFR-1 siRNA (Sirna-027, AGN 211745) has been shown to be well tolerated and to stabilize or improve visual acuity and foveal thickness.[94] However, to make further progress in the development of neovascular AMD treatment, it could be beneficial to address aspects of the disease other than angiogenesis. This might include strategies to protect neurons from hypoxia, oxidative stress, or both. One of the proteins upregulated by the latter 2 conditions is RTP801, overexpression of which can lead to neuronal damage.[95,96] An MOE-modified siRNA targeting RTP801 (PF-04523655) has been developed and subsequently tested in clinical trials (Table V and see Table E1).[41,42] It has been demonstrated to be safe and well tolerated on intravitreal injection. Furthermore, a higher average gain in visual acuity was observed in patients with subfoveal CNV caused by neovascular AMD treated with the combination of PF-04523655 and ranibizumab (an mAb neutralizing VEGF) when compared with ranibizumab alone. This observation might reflect the independence and potential complementarity of the mechanisms of action between the 2 drugs. However, one cannot exclude that the beneficiary effects of PF-04523655 are partly mediated by its influence on VEGF expression through the m-TOR pathway.[42] In addition, PF-04523655 has been tested in patients with diabetic macular edema, demonstrating improvement in visual acuity in this group.[43]

Antisense approaches addressed neovascularization in the cornea as well. Aganirsen (GS-101), a PT ODN–targeting insulin receptor substrate 1 (IRS-1) delivered as eye drops, has been shown to be safe and well tolerated[44] and to exert beneficial effects, such as inhibition, reduction/regression, and/or stabilization, on progressive corneal neovascularization secondary to keratitis or keratouveitis.[45,46] These were accompanied by a lower corneal transplantation need and improved quality of life observed in a recent phase III clinical trial (Table V and Table E1).[46] IRS-1 is a cytoplasmic docking protein that mediates downstream signaling of insulin and insulin-like growth factor 1. The insulin-like growth factor 1 system is thought to be involved in angiogenesis, possibly through regulation of VEGF or other cytokines.[97,98] In preclinical studies antiangiogenic effects of IRS-1 knockdown with aganirsen were indeed accompanied by decreased expression of IL-1β, VEGF, or both.[99,100] Interestingly, formulated as a skin ointment and applied in patients with plaque psoriasis, aganirsen led a to reduced lesion size, diminished expression of IRS-1 and VEGF, and a decrease in T-cell infiltration and keratinocyte proliferation (see Table E1).[101]

Also, other ocular conditions have been targeted by antisense approaches. Eye drops containing SYL040012, an siRNA against the β_2-adrenergic receptor (ADRB2), were found to be safe and well tolerated and to reduce intraocular pressure in healthy volunteers, and thus they can be expected to be further tested in patients with glaucoma.[47] Targeting connexin 43 with the topical gel-formulated ODN Cx43AsODN in subjects with persistent severe chemical and/or thermal ocular burns unresponsive to established therapy led to a rapid reduction in inflammation, as well as a complete and stable corneal re-epithelialization (Table V and see Table E1).[48] Taken together, along with the trials on CMV retinitis discussed above,[29-31] these studies indicate that the eye represents another important organ with diseases accessible to local therapy with antisense strategies.

MISCELLANEOUS

In other disease areas important clinical trials have also been conducted recently (Table VI and Table E1). The antisense approaches studied there included simple or modified RNA/DNA oligonucleotides, siRNA, or a ribozyme, and with the exception of the trial on transthyretin amyloidosis, in which lipid nanoparticle encapsulation was applied, they were delivered to the patients as naked molecules.

Familial amylotrophic lateral sclerosis (ALS),[102] transthyretin amyloidosis,[49] pachyonychia congenita,[103] and Duchenne muscular dystrophy (DMD)[50-52] all have a genetic background. In the case of DMD, antisense treatment aims to restore dystrophin-encoding target gene expression by means of correction of detrimental effects of the mutations on mRNA splicing, leading to formation of an internal (premature) stop codon. This is achieved by affecting splicing in a way that reintroduces an open reading frame, enabling production of a quasinormal (internally truncated) protein, and thus the course of the disease can be alleviated.[104] In patients with ALS, transthyretin amyloidosis, or pachyonychia congenita, in turn,

the biochemical goal of the therapy is to (selectively) reduce the undesired expression of (mutant) mRNA, specifically superoxide dismutase 1, soluble; transthyretin; or keratin 6A, type II transcripts.[49,102,105,106] Also, in patients with myasthenia gravis, the antisense treatment targets a genetically undetermined, so-called read-through unwanted splicing variant of acetylcholinesterase mRNA, overproduction of which contributes to the pathophysiology of the disease. Interestingly, this last molecule was also found to act as a Toll-like receptor (TLR) 9 ligand, which seems to further boost its primary antisense effect.[107-110] Differing by means of administration, ranging from oral through subcutaneous, intravenous, intramuscular, and intrathecal to intradermal, the antisense molecules against DMD (PRO051, drisapersen, GSK2402968), myasthenia gravis (EN101, monarsen), ALS (ISIS 333611), transthyretin amyloidosis (ALN-TTR01/ALN-TTR02), and pachyonychia congenital (TD101) all produced no serious adverse effects, showed a good safety profile, and, except for a molecule against ALS not yet tested for this kind of effect, demonstrated promising biochemical and/or clinical results in terms of efficacy. This creates a chance for some other disorders, the pathology of which is based on (mutation-determined) gene overexpression. However, an application of the antisense strategy successfully used for DMD might be more difficult in case of some other genetically determined deficiencies in protein synthesis resulting from introduction of the premature stop codon, eg, Marfan syndrome or natural anticoagulation defects.[111-113] This is because in these or similar conditions, restoration of fully and not only partially functional protein expression might be more crucial. While discussing the application of antisense approaches in patients with neuromuscular or neurologic disorders, such as ALS or myasthenia gravis, a very recent trial was conducted in patients with relapsing-remitting multiple sclerosis. A modified ODN ATL1102 targeting integrin, the alpha 4 gene (also known as CD49D or the alpha subunit of the very late antigen 4 receptor), was tested. The results of this study showed very promising results in terms of multiple sclerosis activity reduction and general good tolerance of the treatment.[53]

Although multiple novel modalities of venous thromboembolism prevention have been developed lately, the risk of iatrogenic hemorrhage remains a concern with anticoagulation therapy,[84,114] thus making a space for a new ODN targeting coagulation Factor XI (ISIS 416858) that proved to be both clinically effective and safe with regard to uncontrolled bleeding.[54] Not only venous system but also arterial bed disorders, specifically coronary arteries affected by atherosclerosis, have been addressed recently by using antisense therapy. Two independent antisense molecules against c-myc mRNA synthesized from the overlapping region of the gene demonstrated a good safety profile on intracoronary delivery in patients with coronary artery disease, although one of them produced no clear clinical benefits in terms of unfavorable remodeling and restenosis after stent implantation.[115-117]

CURRENT CONSIDERATIONS AND FUTURE PERSPECTIVES

Two antisense drugs have been approved by the FDA thus far, fomivirsen (in 1998) and mipomersen (in 2013),[38,80] and the number of clinical studies on this group of medications has increased over the last 5 to 10 years. Analyzing publication dates of clinical trial papers on antisense drugs, as summarized in Table E1, makes it clear that more than 75% of them were published after 2005 (inclusive) and more than half after 2010 (inclusive). Also, the success of mipomersen seems to be a result of those 2 waves, especially the latter. Considering the increasing number of published clinical studies (and most probably those ongoing as well), one could possibly expect that further antisense drugs will soon become available on the market.

There are several reasons for this optimism. The last few years yielded a variety of modifications in the chemistry of antisense drugs, resulting in improved binding affinity and stability.[6,7] On the other hand, the more modified antisense drugs become, the less natural the products of their degradation might be, which was one of their original advantages over chemical compounds. Furthermore, many studies reported successful application of locally administered antisense therapy accompanied by an advantageous systemic pharmacokinetic profile (ie, limited appearance and rapid clearance of drugs from circulation). From this point of view, it would not be necessary to increase the stability of locally administered antisense drugs over a certain limit, especially because their regional effect can be sustained by more frequent application. Naturally, enhanced stability and a lasting presence in the circulating blood would be beneficial for drugs administered systemically. However, some of those drugs can induce adverse effects, such as liver steatosis and flu-like symptoms in the case of mipomersen. Indeed, side effects, especially liver-related effects, were the most important reason for lack of European Medicines Agency approval for mipomersen.[118]

In general, adverse effects of antisense drugs can be grouped based on (1) route of delivery an place of action (local effects, eg, local injection-site reactions, ocular surface discomfort after administration of eye drops, and itching after application of skin ointment, or systemic effects, eg, flu-like symptoms, liver toxicity, or prolongation of activated partial thromboplastin time); (2) type of carrier; and (3) character of the antisense molecule itself (off-target effects through silencing of additional genes or modulation of other targets, eg, TLRs, resulting from biochemistry of the molecule[4,6,7]). Intriguingly, in some cases the effect mediated through TLRs can enhance the primary gene-silencing action of the antisense drug.[110]

Delivery of antisense molecules represents another important issue. They are thought to be primarily taken up by cells through endocytosis.[119] Interestingly, in all clinical trials analyzing the effects of locally delivered (inhalation by means of oral nebulization, nasal spray, skin ointment, enema, intravitreal injection, and many others) antisense drugs and a huge majority of studies investigating those administered systemically (eg, intravenous infusion, oral tablets, and subcutaneous injection), as identified by our search, including those successful in term of therapeutic efficacy, candidate molecules are administered in the form of naked compounds (see Table E1). Because *ex vivo* uptake of antisense molecules typically requires additional effort, such as use of transfection reagents, one might speculate that some specific and/or unspecific mechanisms supporting their cellular uptake exist *in vivo*. Still, to (further) improve the delivery of antisense compounds, especially those administered systemically, additional techniques are being applied, eg, lipid nanoparticle encapsulation or covalent linking to molecules potentially offering additional benefit of targeting specific cells, tissues, or organs.[5,39,49] In addition, a strategy of harvesting human cells, their *ex vivo* retroviral or lentiviral transduction

with vectors expressing antisense molecules, and subsequent reinfusion, potentially guaranteeing long-term silencing effects, has been developed for use in patients with HIV.[25-27,72,73]

Taken together, promising efficacy results and reasonable tolerance, the latter especially evident in case of local therapies, open the perspective to increase the pipeline of antisense-based drugs for the treatment of allergic diseases. These disorders are typically restricted to certain organs (the upper and lower respiratory tract, skin, gut, and eyes) not requiring systemic administration of medications, and there is still a strong medical need for novel causative acting drugs in this field.

CONCLUSIONS

In conclusion, the big advantage of antisense strategies is their target specificity. This makes the clinical application of antisense technologies highly attractive at a time when research on the pathogenesis of major diseases leads to identification of underlying molecular pathways and thus novel targets for therapeutic intervention. On the other hand, antisense technologies must overcome a number of challenges, including *in vivo* stability, drug delivery, and potential side and off-target effects. The recently published trials in disease areas, including infectious disease, chronic inflammatory conditions (including allergy and asthma), metabolic diseases, and several neurologic disorders, clearly indicate the increasing interest in the clinical application of antisense strategies. More and longer clinical trials are certainly warranted in most of these conditions to make antisense-based therapies clinically available. However, today it can be concluded that antisense-based therapies have the great opportunity to establish themselves as an additional column of therapeutic options in addition to biologicals and small molecules.

We thank Mrs Inge Schmidt and Mrs Jorun Braun for their secretarial and editorial assistance and Mrs Nesibe Akdağ for her help with figure preparation.

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