REVIEW

Institut für Pharmazeutische Chemie, Philipps-Universität Marburg, Germany

Chemical modification of nucleic acids as a key technology for the development of RNA-based therapeutics

A. Grünweller, R. K. Hartmann

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PD Dr. Arnold Grünweller, Prof. Dr. Roland K. Hartmann, Institut für Pharmazeutische Chemie, Philipps-Universität Marburg, Marbacher Weg 6, 35032 Marburg, Germany
gruenwel@staff.uni-marburg.de, roland.hartmann@staff.uni-marburg.de

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RNA-based effector molecules (nucleic acid effectors) are important tools in molecular medicine because they offer a strategy to address therapeutically interesting targets that are not "druggable" with classic small molecule inhibitors. However, for *in vivo* applications, RNA-based effectors require specific chemical modifications to improve their stability and pharmacokinetic properties, as well as to minimize toxic and unspecific off-target effects.

1. Introduction: Why is RNA relevant in medicine?

Since the sequence of the human genome has been published in 2001, a multitude of non-coding RNAs (ncRNA) with novel functions have been discovered, thus moving RNA research to the center of cell biology and molecular medicine. The breathtaking progress in high-throughput next generation sequencing (NGS) methods has enabled us to comprehensively explore the transcriptome of a given cell under normal and pathological conditions. The analysis of the functional relevance of nc-RNAs for pathological processes is currently a major research focus. Some of the most important, newly identified RNA classes are microRNAs (miRNAs), small interfering RNAs (siRNAs), PIWI-interacting RNAs (piRNAs, interacting with PIWI proteins during spermatogenesis), enhancer-accociated RNAs (eRNAs), circular RNAs (circRNAs) and long non-coding RNAs (lncRNAs) (Esteller 2011; Hansen et al. 2013). The functional significance of several ncRNAs is still not clear, but especially for miRNAs and lncRNAs some basic and disease-related regulatory mechanisms have been described, which add a new layer of complexity to the field of regulated gene expression (Rinn and Chang 2012). In turn, by taking the biology of regulatory RNAs into account, we are about to better understand the mechanisms leading to cancer, neurodegeneration or metabolic diseases, which opens up new perspectives for the development of RNA-based therapeutic strategies to treat these pathologies. One of these new therapeutic options is called miRNA replacement. This applies to conditions where a reduced level or the complete loss of a regulatory miRNA is causative for a specific disease and can be cured by delivery of a synthetic miRNA mimic into the respective tissue or organ. The use of such miRNA mimics has been successfully tested in different mouse tumor models (Ibrahim et al. 2011).

On the other hand, cellular RNAs can be addressed as therapeutic targets. This pertains to pathologically overexpressed mRNAs, miRNAs or lncRNAs that can be inhibited by siRNAs (mRNAs and lncRNAs) or antisense oligonucleotides (AONs). In general, target RNAs can either be blocked sterically or their enzymatic degradation can be induced (Watts and Corey 2012). Another important option is the use of RNA as a biomarker in the field of medical diagnostics. Here, a promising approach is the detection of specific miRNAs in the blood of patients as a gentle and early diagnosis of various diseases (Schwarzenbach et al. 2014).

For the application of therapeutic RNA effectors it is essential to prevent their rapid degradation by nucleases in the blood or in living cells. This is approached by incorporating chemical modifications that increase the stability of RNA effectors without compromising their functionality. Another challenge is the prevention of immunostimulatory effects triggered by nucleic acid therapeutics. Here, efforts in basic research are still needed to better understand the underlying molecular principles. Nevertheless, significant improvements toward minimizing such side effects were achieved by introducing strategic chemical modifications at specific positions of RNA effectors, which has largely improved the prospects for the clinical application of nucleic acid-based drugs.

2. Chemically modified nucleic acids: state of the art and challenges

A series of innovations in nucleic acid chemistry, developed in the last two decades, now allows researchers to fine-tune the properties of nucleic acid effectors. This includes stability improvements up to full nuclease resistance, increased binding affinity and specificity for a given RNA target and a reduction of unwanted immunostimulatory effects (Deleavey and Damha 2012). A better understanding of miRNA biogenesis and their mode of action has made it possible to minimize those "off-target" effects" of siRNAs that result from unintended miRNA-like effects of siRNAs at other mRNAs (Bramsen et al. 2010).

Overall, the safety issue of therapeutically relevant nucleic acid effectors has significantly improved during the last years. However, additional pharmacokinetic aspects are to be considered for therapeutic use. For example, biodistribution, metabolic stability, rate of clearance, residence time in the organism and, in particular, cellular uptake are of crucial importance. Cellular

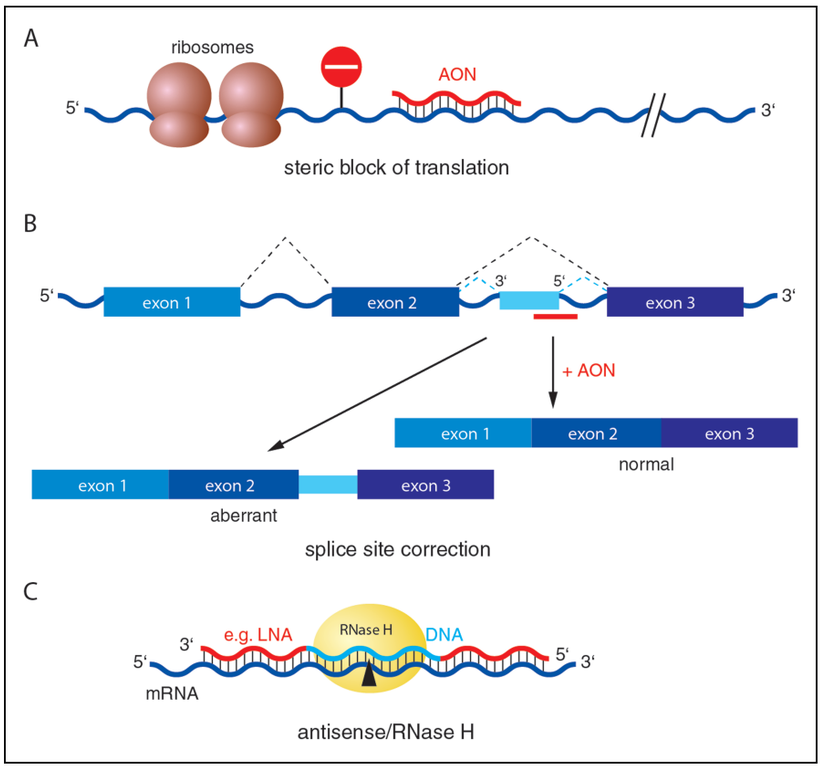

Fig. 1: Illustration of antisense effects. (A) The binding of an antisense oligonucleotide (AON) to a complementary target mRNA results in a steric blockage of protein biosynthesis. (B) Utilization of an unwanted, disease-relevant splice site is blocked by an AON. (C) Design of a gapmer AON containing at least 6 DNA nucleotides in the center for recruiting the endogenous endonuclease RNase H to the DNA:RNA hybrid. As a result, the target RNA is cleaved by RNase H and subsequently degraded by cellular exonucleases. LNA, locked nucleic acid.

uptake is a major topic as cell membranes are biological barriers that are difficult to overcome by nucleic acids. Although there are a number of approaches to improve cellular uptake, for example by chemical modifications or formulations of nucleic acid effctors (see Merkel et al. and Aigner et al. in this issue), the development of specific, efficient and non-toxic delivery strategies is still the "bottleneck" on the way to the clinical application of nucleic acid effectors.

3. Therapeutic application of chemically modified nucleic acids: Antisense strategies and RNA interference

Chemical nucleic acid modifications are relevant to antisense oligonucleotides, RNAi effectors, aptamers, DNA/RNAzymes and immunostimulatory nucleic acids. Here we put our focus on antisense and RNAi strategies owing to their central role in the research and development of nucleic acid therapeutics.

Antisense oligonucleotides (AONs) can be divided into two categories: (1) AONs that cause a steric block and (2) AONs that mediate target RNA cleavage. A steric block AON interferes with the binding of proteins or ribonucleoprotein complexes to the target RNA or prevents the processivity of the ribosome during mRNA translation (Fig. 1A). Correction of aberrant splicing can be achieved by blocking the falsely used splice site with an AON, thus forcing the splicing machinery to use the canonical splice site (Fig. 1B) (Grünweller and Hartmann 2007).

The second type of AON recruits endogenous RNase H to a double strand consisting of the AON bound to the target RNA (Fig. 1C). RNase H cleaves the RNA strand in RNA:DNA duplexes. A few DNA analogs that structurally mimic a DNA strand can be used instead of DNA residues (Damha et al. 1998).

Another variation is to confine the chemical modifications to the terminal regions of the AON while keeping a central region of at least six DNA nucleotides, known as the "Gapmer" design (Grünweller et al. 2003). Gapmers combine the beneficial properties of modified AONs with the ability to recruit endogenous RNase H to the RNA:DNA hybrid region for cleavage of the target RNA strand.

RNA interference (RNAi) is a cellular mechanism whose central feature is the production of short double-stranded RNAs (dsRNAs) that are loaded into an effector complex termed "RNA-induced Silencing Complex" (RISC). These short ds-RNAs naturally derive from processing of longer precursors which may be exogenous RNAs or endogenous transcripts. After RISC loading, one RNA strand is degraded and the remaining one guides the RNAi machinery to the target mRNA(s) to suppress protein translation (Abb. 2) (Grünweller and Hartmann 2005; Fabian et al. 2010; Ha and Kim 2014). In the case of siRNAs, the short RNA duplexes have a length of ~ 19 base pairs (bp) with 2 nucleotide (nt) overhangs at the respective 3'-ends. The architecture of miRNA duplexes is basically the same, except that the duplex is interrupted by small bulges and internal loops. While siRNAs are normally derived from foreign dsRNAs (e.g. of viral origin), miRNAs are encoded and expressed in the cells themselves. The structural design of miRNAs differs from that of siRNAs in that the latter are fully complementary to their target mRNAs, whereas miRNAs bind their mRNA target sites (predominantly in mRNA 3'-UTRs) only by partial complementarity (Fig. 2 and 3).

Both types of effector RNAs are loaded into similar RISC complexes where the so-called passenger or sense strand is cleaved and removed, while the guide or antisense strand (~ 22 nt) remains bound. The guide strand leads RISC to the

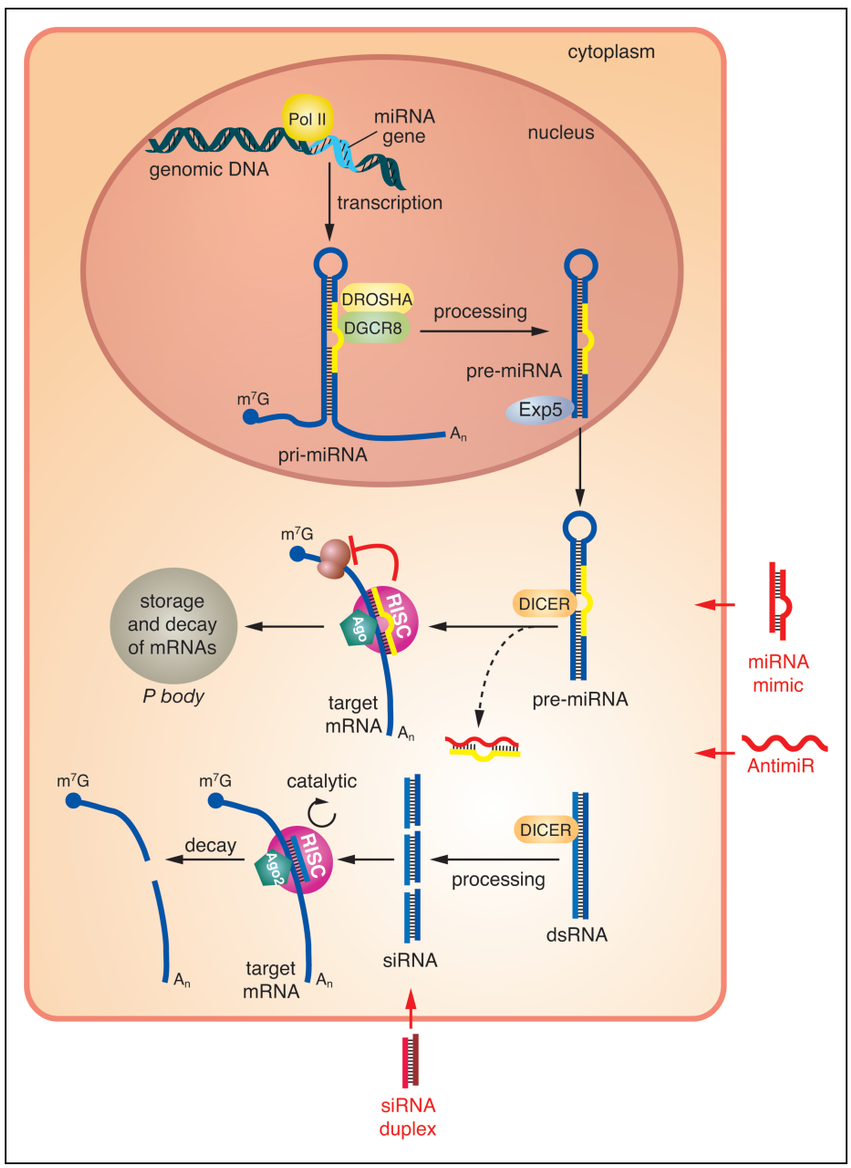

Fig. 2: RNA interference (RNAi) in mammalian cells and intervention by celluler uptake of siRNA duplexes, miRNA mimics and AntimiRs. In the cytosolic siRNA pathway, usually long exogenous (e.g. of viral origin) RNA duplexes are processed by the RNase Dicer into short siRNA duplexes (19 bp) with 2-nt overhangs at the 3'-ends, which are then incorporated into the RNA-induced silencing complex (RISC). Within RISC, one of the two siRNA strands is degraded, while the other one, the guide strand, is used to target RISC to complementary mRNA sequences that are cleaved by the endonuclease Ago2 to induce mRNA decay. This is a catalytic process (one RISC loaded with an siRNA guide strand can cleave many complementary mRNA molecules), explaining the high efficiency of RNAi. In the endogenous miRNA pathway, genomically encoded miRNAs are usually transcribed in the nucleus by RNA polymerase II. After processing of these so-called pri-miRNAs by Drosha and DGCR8, the resulting pre-miRNAs are transported from the nucleus to the cytoplasm through the nuclear export factor exportin 5. Here, the same processing steps take place as described for the siRNA pathway. However, siRNA and miRNA pathways differ after guide strand selection: whereas siRNAs are fully complementary to the target mRNA and induce its cleavage, miRNA strands guide the RISC primarily to the 3'-UTR of their target mRNAs where they form only partial base pairing interactions. The prevailing biological miRNA effects are inhibition of the 5'-cap-dependent initiation of protein biosynthesis and/or induction of mRNA transfer into cytoplasmic "Processing (P) bodies", where the mRNAs are stored or degraded; m^7G: 7-methyl-guanosine-5',5'-triphosphate cap at the 5' end of mRNAs; A_n: poly (A) tail at the 3'-end of mRNAs.

complementary region in the target mRNA that can either be translationally inhibited (miRNA) or cleaved (siRNA) by the endonuclease argonaute (Ago) to induce mRNA decay (Grünweller and Hartmann 2005). MiRNAs also trigger a transfer of the bound mRNAs into cytoplasmic structures termed "processing (P) bodies", in which the mRNAs are stored or degraded (Fig. 2). RNAi is a very efficient and straightforward method to inhibit mRNA translation, because one RISC can inactivate multiple target mRNAs and siRNAs can in principle be designed against any mRNA. MiRNAs can also be introduced into cells as synthetic miRNA mimics (Fig. 2) to reduce the expression of their target mRNAs. Owing to imperfect base pairing with mRNAs, miRNAs usually act on multiple target mRNAs.

Expression profiles of miRNAs are usually altered in pathological states of cells. For example, the levels of miRNAs regulating the production of tumor suppressor proteins are often increased in cancer cells (O'Donnell et al. 2005; Olive et al. 2009; Volinia et al. 2006). Such pathologically overexpressed miRNAs can be targeted by AONs that are referred to as AntimiRs (Fig. 2).

Generally, specific chemical modifications introduced into siRNAs, miRNA mimics and AntimiRs can improve cellular uptake, target specificity and the profile of side effects. Such adverse side effects, also termed "off-target" effects, include:

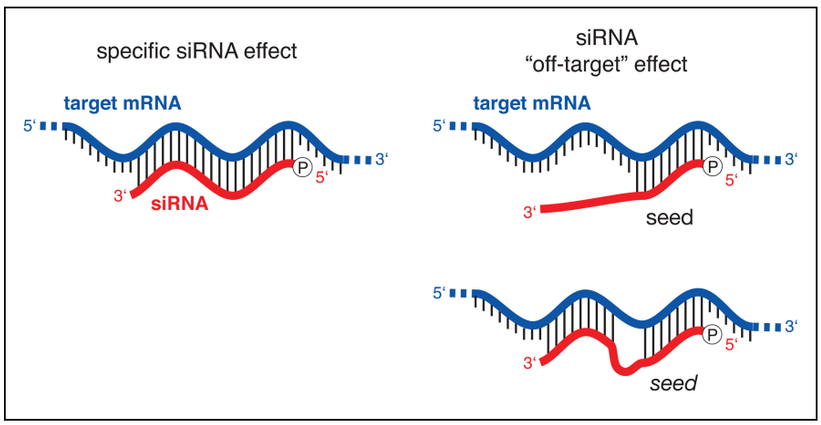

Fig. 3: Specific siRNA effect (left) and siRNA "off-target" effects, in which the siRNA guides RISC like a miRNA to 3' untranslated regions in other mRNAs. The minimal requirement for such a miRNA effect is that the siRNA strand shows continuous base pairing with the mRNA in its seed region (nucleotide positions ~ 2-8 at the 5'-end of the siRNA) (Bartel 2009).

(i) non-specific immunostimmulatory effects,
(ii) miRNA-like effects of siRNAs at other non-target mRNAs (mediated *via* the minimal seed pairing; Fig. 3), and
(iii) a saturation of the cellular RNAi machinery by therapeutic siRNAs and miRNA mimics, which may also give rise to adverse effects.
In addition, there is the possibility that RISC complexes to some extent select and retain the passenger instead of the guide strand, which could weaken the strength of the specific RNAi effect and may increase the risk of additional non-specific miRNA-like effects (Bramsen et al. 2007, 2014).

4. Where to modify RNA?

Nucleic acids can be modified at the phosphate, the sugar or (less often) at the heterocyclic base (Fig. 4 A). Strategic modifications at the backbone, primarily on the sugar, can potentially improve stability, specificity, cellular uptake and/or biodistribution of nucleic acid effectors. Particularly the 2'-OH group of the ribose, which makes RNA molecules much more reactive and unstable than DNA, is well suited for chemical modifications. Importantly, 2'-OH modifications are usually highly compatible with the function of AONs and siRNAs (Schirle and MacRae 2012) and, for example, 2'-*O*-methylation is further able to suppress undesirable immunostimulatory effects (Kariko et al. 2005; reviewed in Bramsen et al. 2014). Another option is coupling of chemical groups with e.g. lipophilic or cationic properties to the ends of nucleic acid effectors to improve cellular uptake. Circulation time, biodistribution, endosomal release and cellular localization of nucleic acid effectors can be modulated by conjugations as well (Soutschek et al. 2004; Wolfrum et al. 2007; reviewed in Aigner 2008, and Bramsen et al. 2014).

5. Examples for nucleic acid modifications

Phosphorothioates (PS; Fig. 4 A), first generation modifications that are still in use, can be cost-effectively incorporated into chemically synthesized nucleic acids. A potential disadvantage of PS-modified oligonucleotides is their often observed slight reduction in RNA binding affinity (Kurreck 2003). This may be compensated by higher oligonucleotide concentrations, although this may entail an increase in adverse side effects. Moreover, generally increased non-specific binding of PS-modified nucleic acids to proteins may cause toxic side effects. On the other hand, enhanced non-specific binding of PS-modified nucleic acids to serum albumin could be advantageous as it may lead to prolonged blood circulation, a more favorable biodistribution and a more efficient cellular uptake in particular (Levin 1999; Geary et al. 2002, 2015). In some cases these properties seem to obviate the need for complex nanoparticles in nucleic acid effector delivery. The therapeutic relevance of PS modifications is illustrated by FDA approval (in 1998) of the drug Fomivirsen, a PS-AON used as an antiviral agent for the treatment of opportunistic cytomegalovirus (CMV) infections in patients with AIDS (Yamamoto et al. 2011).
Another option beyond substituting the 2'-OH group is to change the scaffold of the sugar moiety, e.g. by replacement of the ribose with other 5- or 6-membered ring structures. Moreover, introduction of a methylene bridge between the 2'-oxygen and the 4'-carbon rigidifies the sugar structure (Grünweller and Hartmann 2007) (so-called "locked nucleic acids" (LNA), Fig. 4 B). The best characterized 2'-substitutions are 2'-*O*-methyl (2'-OMe), 2'-fluoro (2'-F) and 2'-*O*-(2-Methoxyethyl) (2'-O-MOE) modifications (Fig. 4 B). Their incorporation is compatible with standard methods of DNA and RNA synthesis, thus facilitating the routine synthesis of AONs with modifications at selected positions (so-called mixmers) including combinations of different modifications. In general, 2'-ribose modifications increase nuclease resistance and 2'-OMe and 2'-F modifications also slightly enhance the affinity for the RNA target. A combination of 2'-OMe and 2'-F modifications in siRNA effectors was reported to significantly increase knockdown efficacy in a mouse model (Morrissey et al. 2005a, b). As mentioned above, 2'-OMe modifications are able to alleviate unwanted immunostimulatory effects, thereby increasing drug tolerability and therapeutic index (Bramsen et al. 2014).
2'-F-ANA-modified nucleic acids adopt a sugar conformation similar to DNA, enhance nuclease resistance, stabilize base pairing with target RNAs, enable cleavage of RNA by RNase H in corresponding 2'-F-ANA:RNA duplexes and facilitate cellular uptake (Deleavey and Damha 2012).
LNA is structurally more related to RNA than DNA and the melting point (Tm) of an LNA:RNA duplex is increased by several degrees Celsius per incorporated LNA nucleotide. To our knowledge, LNA-modified oligonucleotides are currently the most potent nucleic acid effectors. Beyond their exceptional duplex stabilization and largely enhanced nuclease resistance, LNA effectors also combine excellent sequence specificity with usually low toxicity (Doessing and Vester 2011; Burdick et al. 2014). For these reasons several LNA-modified nucleic acid effectors are currently in clinical trials (http://www.roche.dk).

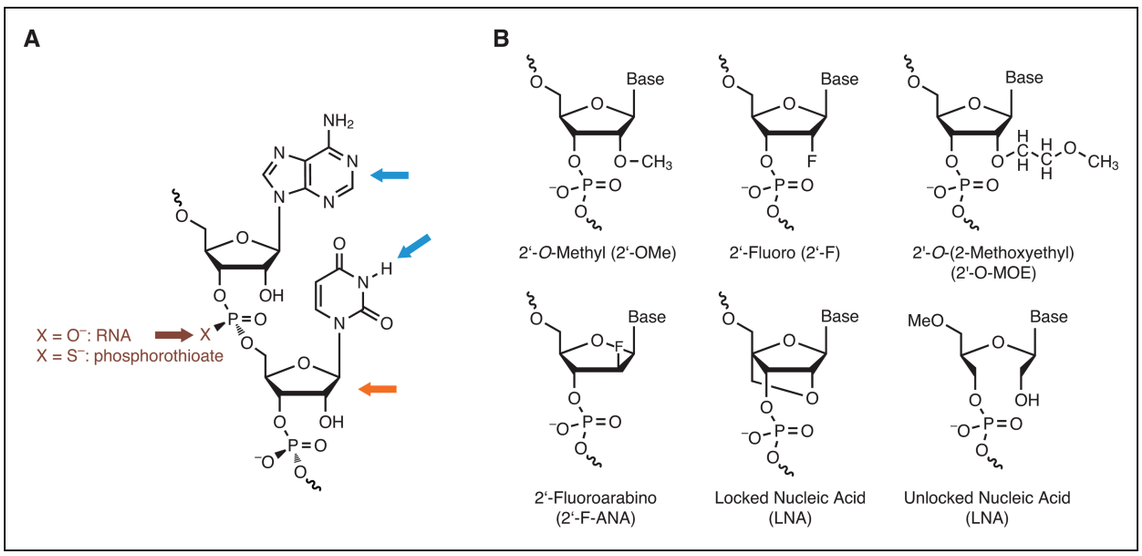

Fig. 4: (A) Chemical nucleotide modifications (blue arrows: heterocyclic base, orange arrow: sugar, brown arrow: phosphodiester). In phosphorothioates, one of the two non-bridging oxygens of the phosphodiester is replaced by sulfur; here, the negative charge is mainly localized on the sulfur while it is equally distributed among the two non-bridging oxygens in phosphodiesters. (B) Promising and frequently used modifications of nucleic acid effectors.

As mentioned above, alterations of the heterocyclic bases are more rarely applied, but can be used to reduce off-target effects at other mRNAs or due to binding to non-RISC proteins, such as ADAR (adenosine deaminase, RNA-specific), protein kinase R (PKR) or TLRs (Peacock et al. 2011). It was recently shown that specific base modifications at the guide strand 5'-end of siRNAs can improve siRNA efficacy by optimizing the interaction with human Argonaute-2 (Onizuka et al. 2013).

6. Examples for the application of modified AONs and siRNAs

The causative role of aberrantly expressed ncRNAs in various pathologies calls for strategies to re-adjust their cellular levels. Overexpressed miRNAs and lncRNAs are target molecules of particular interest for therapeutic interventions. For short ncRNAs, such as miRNAs, specific inhibition is challenging as they provide only a small sequence space for complementary base pairing. Another complication arises from the fact that the essential interaction with target mRNAs occurs *via* a so-called seed region that only comprises the first 6-8 nt of the miRNA's 5'-end. Since in many cases several miRNAs share an identical seed sequence (miRNAs belonging to the same miRNA family), they can potentially bind the same target mRNAs despite differences in their central and 3'-proximal regions. In view of this functional redundancy of miRNA family members, it appears useful to develop inhibitors against entire miRNA families.

6.1. AntimiRs to inhibit miRNAs

MiRNA inhibition by AntimiRs was first successfully tested in mice employing the so-called AntagomiR design. AntagomiRs, directed against the entire miRNA sequence (~22 nucleotides), are 2'-OMe- and PS-modified AONs with a conjugated cholesterol moiety at the 3'-end (Krützfeldt et al. 2005). This design protects AntagomiRs against nuclease attack and improves cellular uptake by virtue of the conjugated cholesterol.
2'-O-MOE- and PS-modified AntimiRs targeting the liver-specific miR-122 also proved to be effective in an *in vivo* mouse model, resulting in reduced serum cholesterol levels (Esau et al. 2006). *In vivo*, continuous 2'-F modification in combination with a PS backbone and two terminal 2'-O-MOE modifications on each end of a 23-meric AntimiR was shown to be particularly favorable (Davis et al. 2009). For the inhibition of miRNAs and particularly miRNA families, LNA-modified AntimiRs are to be considered. Owing to the extraordinary duplex stabilization mediated by LNA it is possible to design extremely short LNA AntimiRs (minimally 8-mers, see below) that specifically bind to their target RNA(s). A PS-modified LNA/DNA mixmer with a length of 15 nt is currently tested in clinical trials. This AntimiR, termed Miravirsen® (Roche Innovation Center Copenhagen A/S) which is also directed against miR-122, significantly reduced hepatitis C virus replication in liver cells (Elmén et al. 2008; Lennox and Behlke 2011; Lindow and Kauppinen 2012). In addition, Miravirsen® lowers serum cholesterol levels by about 25%. Of particular interest are LNA-PS AntimiRs with a length of 8 nt that were named "tiny LNAs" (Obad et al. 2011). They are directed against the seed region of miRNAs and therefore inhibit all miRNAs of a respective family. As another favorable feature, short LNA-PS AntimiRs can be delivered as functional molecules into cells of some tissues (especially into the liver) without any formulation ("naked" delivery) (Stein et al. 2010). Tiny LNA AntimiRs might therefore gain central importance in therapeutic applications. Finally, short LNA AntimiRs with a natural phosphodiester backbone and a length of 12-14 nt, which we termed *antiseeds*, are also able to block miRNA families. Antiseeds, packed up in polymeric nanoparticles consisting of a branched low molecular weight polyethylenimine, can be delivered to target cells and released from these nanoparticles in a functional form after cellular uptake (Thomas et al. 2012). Such nanoparticles may turn out to be a genuine alternative for the delivery of LNA AntimiRs into tissues that are not reachable by "naked" AntimiRs.

6.2. Gapmers to inhibit mRNAs and lncRNAs

For antisense inhibition of long RNAs (mRNAs and lnc-RNAs), modified gapmers (Fig. 1 C) are currently considered as the most efficient molecules (apart from siRNAs in the case of mRNA knockdown by RNAi). Gapmers usually have an internal region of DNA nucleotides flanked by modified nucleotides at both ends to protect the gapmer against degradation by nucleases.

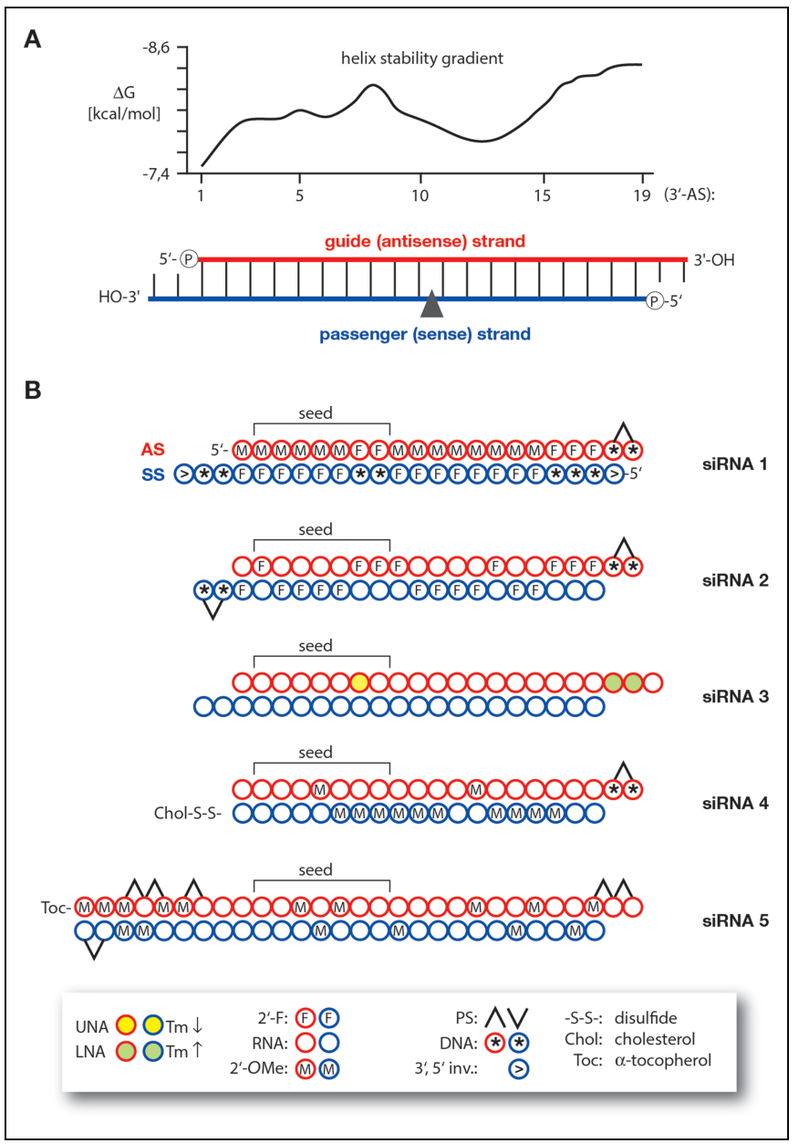

Fig. 5: Design and structural properties of siRNAs and examples of chemically modified siRNAs with improved properties. (A) asymmetric stability gradient between the ends of the siRNA duplex, which favors selection of the guide strand by RISC (modified based on Khvorova et al 2003). Bottom: siRNA duplex with Ago cleavage site in the passanger strand indicated by the black triangle. (B) Examples of chemically modified siRNAs that show promising knockdown effects (siRNA 1: Morissey et al. 2005a, b; siRNA 2: Manoharon et al. 2011; siRNA 3: Bramsen et al. 2010 (antisense strand W361); siRNA 4: Chen et al. 2010; siRNA 5: Nishina et al. 2008).

Particularly efficient are LNA gapmers (Grünweller et al. 2003). The entire gapmer (14 to 16-mers, as offered by Exiqon) or the internal DNA sequence may additionally carry PS modifications that are fully compatible with RNase H-catalyzed cleavage of the complementary RNA target strand. Successful inhibition of lncRNAs by gapmers (Michalik et al. 2014) opens up the perspective to explore an entire new class of ncRNAs for therapeutic applications. Also, most AONs that are currently in clinical trials are gapmers. An example is Mipomersen (Kynamro®, formerly ISIS Pharmaceuticals), a 20-meric AON with PS-modifications at all internucleotide linkages, 2'-O-MOE modifications at the first and last five residues and the central ten residues being DNA. Mipomersen is used to treat familial hypercholesterinemia by lowering cholesterol levels *via* inhibition of apolipoprotein B mRNA (Rader and Kastelein 2014). A clinical phase III study has already been completed and the US Food and Drug Administration approved Mipomersen in 2013.

6.3. Chemically modified siRNAs

Several studies in cell culture have shown that siRNAs are more efficient mRNA inhibitors than AONs (Bertrand et al. 2002; Grünweller et al. 2003; Miyagishi et al. 2003), although comparable efficacies of siRNAs and RNase H-recruitung AONs were also reported (Vickers et al. 2003). RNAi-based strategies are therefore still an interesting option, even if the initial euphoria for the therapeutic use of RNAi has been replaced by sober objectivity. For some time, extensive research efforts have been made to chemically modify siRNAs for therapeutic applications such that off-target effects are avoided and pharmacokinetic properties are improved (Bramsen et al. 2014).

Beyond chemical modification, the architectural design of siRNA molecules also plays an important role for their activity. In addition to conventional siRNAs (19 bp and 2 nt 3'-overhang at each end), precursor molecules such as short hairpin RNAs (shRNAs; typically with a duplex length of 21 bp) or 27-bp

siRNAs can be used that are processed to mature siRNAs by the endonuclease Dicer in the cytoplasm of recipient cells. It is believed that such siRNA variants are more effectively incorporated into the cellular RNAi machinery, which is consistent with the observation of increased efficiency (Bramsen et al. 2014). However, there is some evidence that siRNAs > 19 bp enhance the potential to trigger a cellular interferon response (Reynolds et al. 2006). Furthermore, active siRNAs with a (i) shortened passenger strand (aiRNAs, asiRNAs (Sun et al. 2008; Chang et al. 2009), (ii) with a passenger strand consisting of two fragments (sisiRNA, Bramsen et al. 2007) or (iii) single-stranded siRNAs entirely lacking the passenger strand (ss-siRNA) (Martinez et al. 2002; Holen et al. 2003; Hall et al. 2006) have been reported. Such formats exclude passenger strand incorporation into RISC.

Another conceptual variation is the incorporation of destabilizing modifications, such as PS or so-called unlocked nucleic acid residues (UNA; Fig. 4 B), into the 3'-proximal region of the passenger strand to weaken helix stability in this part of the duplex (Bramsen et al. 2010, Fig. 5A). This reduces the inadvertent retention of passenger strands in RISC complexes. By installing a destabilizing UNA modification at position 7 in the seed region of the siRNA guide strand, miRNA-like off-target effects can be efficiently reduced (Bramsen et al. 2010). This local destabilization is neutral to the specific siRNA effect itself, since base pairing with the target RNA takes place over 19 consecutive nucleotides, whereas miRNA-like seed interactions with only 6-8 bp are severely destabilized by the single UNA modification at position 7. Some examples of effective, chemically modified siRNAs are shown in Fig. 5 B.

A problem associated with potential therapeutic applications of siRNAs is the stimulation of our innate immune system. For example, the helical structure of siRNAs is recognized as "foreign" by the membrane-bound Toll-Like Receptor 3 (TLR3), which then triggers the production of cytokines. Activation correlates with the length of the siRNA double strand, and is no longer detectable for siRNAs < 19 bp in the mouse system (Kleinman et al. 2008). However, such findings in mice do not simply apply to humans since the expression of TLR3 is different in the human versus murine immune system (for review see Bramsen et al. 2014). SiRNAs that are delivered *via* the endosomal pathway (e.g. in the form of nanoparticles) into cells can also come into contact with the human endosomal receptors TLR7 and TLR8. These receptors recognize G/U-rich sequence motifs and are able to trigger immunostimulatory effects as well. However, 2'-OMe, 2'-F and also base modifications were shown to significantly reduce siRNA immunogenicity (Morrissey et al. 2005b). 2'-OMe and various base modifications also occur in our own RNAs and are used to distinguish endogenous RNAs from other foreign RNAs that may originate from pathogens.

7. Nucleic acid conjugations

Different transport vehicles are available for the delivery of nucleic acids into cells. This includes viral vectors, cationic liposomes (lipoplexes), dendrimers and cationic polymers (polyplexes) (Grünweller and Hartmann 2005; Aigner 2008). Basically, all these formulations neutralize the negative charges of nucleic acids to allow contact with the negatively charged cell membrane as a prerequisite for cell entry. Nucleic acid effectors can also be derivatized at their termini with functional groups such as "cell penetrating peptides" (CPP), cholesterol or α-tocopherol (Grünweller and Hartmann 2005) to make the cellular uptake across the plasma membrane more efficient. In addition, toxic effects of nucleic acid effectors can be reduced by targeted delivery into specific tissues or cell types. A specific targeting can be pursued by coupling cell type-specific ligands to nucleic acid effectors, such as peptides, antibodies or aptamers (Aigner 2008). In addition, conjugates like bile acids or long-chain fatty acids have been shown to mediate cellular uptake of siRNAs (via interactions with lipoprotein particles, lipoprotein receptors and transmembrane proteins) and to modulate their tissue distribution (Wolfrum et al. 2007). At present, a number of non-viral delivery strategies are being tested in clinical trials for the delivery of nucleic acid effectors. These include, for example, SNALPs (stable nucleic acid lipid particles), DPCs (dynamic polyconjugates), CDPs (cyclodextrin-based polymers) and siRNA-GalNAc (N-Acetylgalactosamine) conjugates (Kanasty et al. 2013).

8. Conclusions

Many disease-relevant targets (e.g. ncRNAs) are at present inaccessible to inhibition by conventional small molecule inhibitors, but are generally accessible *via* nucleic acid-based therapeutic approaches. However, several hurdles have to be overcome on the way to a successful therapeutic application of nucleic acids. The application of non-toxic nucleic acid modifications for a specific adjustment of pharmacokinetic properties and the development of efficient and cell type-specific delivery strategies are the key technologies toward the clinical use of nucleic acid effectors.

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