Eur. J. Biochem. **270**, 1628–1644 (2003) © FEBS 2003 doi:10.1046/j.1432-1033.2003.03555.x

REVIEW ARTICLE

Antisense technologies

Improvement through novel chemical modifications

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Antisense agents are valuable tools to inhibit the expression of a target gene in a sequence-specific manner, and may be used for functional genomics, target validation and therapeutic purposes. Three types of anti-mRNA strategies can be distinguished. Firstly, the use of single stranded antisense-oligonucleotides; secondly, the triggering of RNA cleavage through catalytically active oligonucleotides referred to as ribozymes; and thirdly, RNA interference induced by small interfering RNA molecules. Despite the seemingly simple idea to reduce translation by oligonucleotides complementary to an mRNA, several problems have to be overcome for successful application. Accessible sites of the target RNA for oligonucleotide binding have to be identified, antisense agents have to be protected against nucleolytic attack, and their cellular uptake and correct intracellular localization have to be achieved. Major disadvantages of commonly used phosphorothioate DNA oligonucleotides are their low affinity towards target RNA molecules and their toxic side-effects. Some of these problems have been solved in 'second generation' nucleotides with alkyl modifications at the 2′ position of the ribose. In recent years valuable progress has been achieved through the development of novel chemically modified nucleotides with improved properties such as enhanced serum stability, higher target affinity and low toxicity. In addition, RNA-cleaving ribozymes and deoxyribozymes, and the use of 21-mer double-stranded RNA molecules for RNA interference applications in mammalian cells offer highly efficient strategies to suppress the expression of a specific gene.

Keywords: antisense-oligonucleotides; deoxyribozymes; DNA enzymes; locked nucleic acids; peptide nucleic acids; phosphorothioates; ribozymes; RNA interference; small interfering RNA.

Introduction

The potential of oligodeoxynucleotides to act as antisense agents that inhibit viral replication in cell culture was discovered by Zamecnik and Stephenson in 1978 [1]. Since then antisense technology has been developed as a powerful tool for target validation and therapeutic purposes. Theoretically, antisense molecules could be used to cure any disease that is caused by the expression of a deleterious gene, e.g. viral infections, cancer growth and inflammatory diseases. Though rather elegant in theory, antisense approaches have proven to be challenging in practical applications.

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Abbreviations: AS, antisense; CeNA, cyclohexene nucleic acid; CMV, cytomegalovirus; FANA, 2′-deoxy-2′-fluoro-β-D-arabino nucleic acid; GFP, green fluorescence protein; HER, human epidermal growth factor; ICAM, intercellular adhesion molecule; LNA, locked nucleic acid; MF, morpholino; NP, N3′-P5′ phosphoroamidates; ON, oligonucleotide; PNA, peptide nucleic acid; PS, phosphorothioate; RISC, RNA-induced silencing complex; RNAi, RNA interference; shRNA, short hairpin RNA; siRNA, small interfering RNA; tc, tricyclo; TNF, tumor necrosis factor.

(Received 16 January 2003, revised 19 February 2003, accepted 4 March 2003)

In the present review, three types of anti-mRNA strategies will be discussed, which are summarized in Fig. 1. This scheme also demonstrates the difference between antisense approaches and conventional drugs, most of which bind to proteins and thereby modulate their function. In contrast, antisense agents act at the mRNA level, preventing its translation into protein. Antisense-oligonucleotides (AS-ONs) pair with their complementary mRNA, whereas ribozymes and DNA enzymes are catalytically active ONs that not only bind, but can also cleave, their target RNA. In recent years, considerable progress has been made through the development of novel chemical modifications to stabilize ONs against nucleolytic degradation and enhance their target affinity. In addition, RNA interference has been established as a third, highly efficient method of suppressing gene expression in mammalian cells by the use of 21–23-mer small interfering RNA (siRNA) molecules [2].

Efficient methods for gene silencing have been receiving increased attention in the era of functional genomics, since sequence analysis of the human genome and the genomes of several model organisms revealed numerous genes, whose function is not yet known. As Bennett and Cowsert pointed out in their review article [3] AS-ONs combine many desired properties such as broad applicability, direct utilization of sequence information, rapid development at low costs, high probability of success and high specificity compared to alternative technologies for gene functionalization and target validation. For example, the widely used approach to generate knock-out animals to gain information about

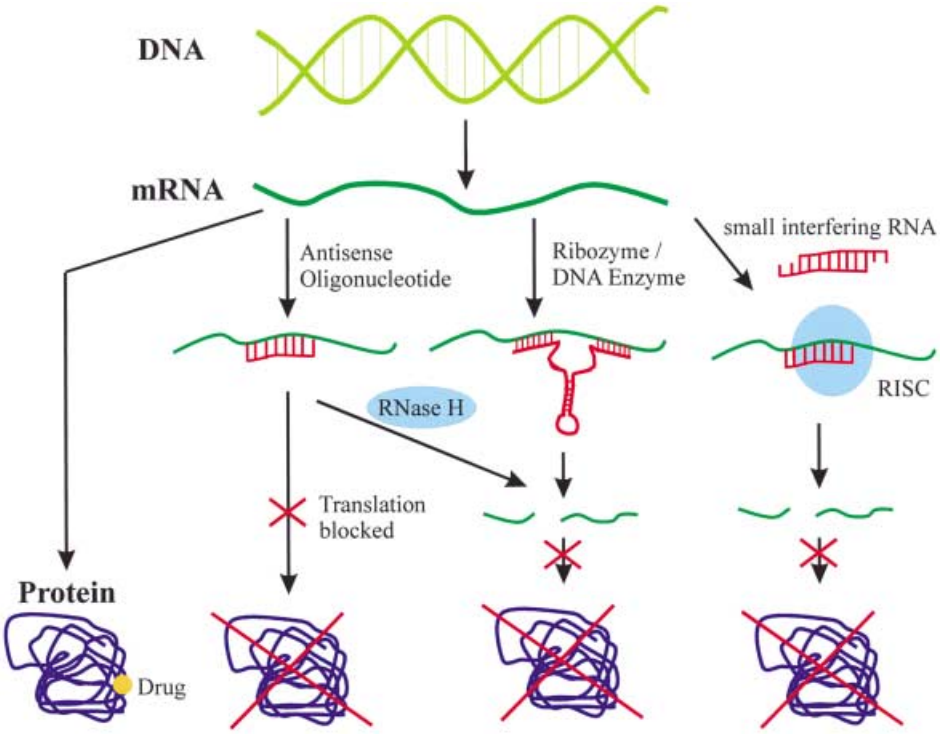

Fig. 1. Comparison of different antisense strategies. While most of the conventional drugs bind to proteins, antisense molecules pair with their complementary target RNA. Antisense-oligonucleotides block translation of the mRNA or induce its degradation by RNase H, while ribozymes and DNA enzymes possess catalytic activity and cleave their target RNA. RNA interference approaches are performed with siRNA molecules that are bound by the RISC and induce degradation of the target mRNA.

the function of genes *in vivo* is time-consuming, expensive, labor intensive and, in many cases, noninformative due to lethality during embryogenesis. In these cases, antisense technologies offer an attractive alternative to specifically knock down the expression of a target gene. Mouse E-cadherin (–/–) embryos, for example, fail to form the blastocoele, resulting in lethality in an early stage of embryogenesis, but AS-ONs, when administered in a later stage of development, were successfully employed to investigate a secondary role of E-cadherin [4]. Another advantage of the development of AS-ONs is the opportunity to use molecules for therapeutic purposes, which have been proven to be successful in animal models.

It should, however, be mentioned that it was questioned whether antisense strategies kept the promises made more than 20 years ago [5]. As will be described in detail below, problems such as the stability of ONs *in vivo*, efficient cellular uptake and toxicity hampered the use of AS agents in many cases and need to be solved for their successful application. In addition, nonantisense effects of ONs have led to misinterpretations of data obtained from AS experiments. Therefore, appropriate controls to prove that any observed effect is due to a specific antisense inhibition of gene expression are another prerequisite for the proper use of AS molecules.

Antisense-oligonucleotides

AS-ONs usually consist of 15–20 nucleotides, which are complementary to their target mRNA. As illustrated in Fig. 2, two major mechanisms contribute to their antisense activity. The first is that most AS-ONs are designed to activate RNase H, which cleaves the RNA moiety of a DNA·RNA heteroduplex and therefore leads to degradation of the target mRNA. In addition, AS-ONs that do not

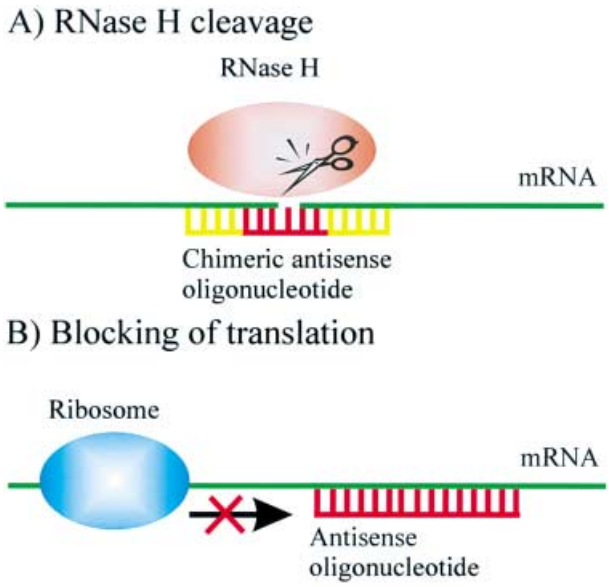

Fig. 2. Mechanisms of antisense activity. (A) RNase H cleavage induced by (chimeric) antisense-oligonucleotides. (B) Translational arrest by blocking the ribosome. See the text for details.

induce RNase H cleavage can be used to inhibit translation by steric blockade of the ribosome. When the AS-ONs are targeted to the 5′-terminus, binding and assembly of the translation machinery can be prevented. Furthermore, AS-ONs can be used to correct aberrant splicing (see below).

Long RNA molecules form complex secondary and tertiary structures and therefore the first task for a successful antisense approach is to identify accessible target sites of the mRNA. On average, only one in eight AS-ONs is thought to bind effectively and specifically to a certain target mRNA [6], but the percentage of active AS-ONs is known to vary from one target to the next. It is therefore possible to simply test a number of ONs for their antisense efficiency, but more sophisticated approaches are known for a systematic optimization of the antisense effect.

Computer-based structure models of long RNA molecules are unlikely to represent the RNA structure inside a living cell, and to date are only of limited use for the design of efficient AS-ONs. Therefore, a variety of strategies have been developed for this purpose (reviewed in [7]). The use of random or semirandom ON libraries and RNase H, followed by primer extension, has been shown to reveal a comprehensive picture of the accessible sites [8,9]. A nonrandom variation of this strategy was developed in which target-specific AS-ONs were generated by digestion of the template DNA [10]. A rather simple and straightforward method providing comparable information about the structure of the target RNA is to screen a large number of specific ONs against the transcript in the presence of RNase H and to evaluate the extent of cleavage induced by individual ONs [11]. The most sophisticated approach reported so far is to design a DNA array to map an RNA for hybridization sites of ONs [12]. Because mRNA structures in biological systems are likely to differ from the structure of *in vitro* transcribed RNA molecules, and because RNA-binding proteins shield certain target sites inside cells, screening of ON efficiency in cell extracts [13] or in cell culture might be advantageous (e.g. [14,15]).

When designing ONs for antisense experiments, several pitfalls should be avoided [6]. AS-ONs containing four contiguous guanosine residues should not be employed, as they might form G-quartets via Hoogsteen base-pair formation that can decrease the available ON concentration and might result in undesired side-effects. Modified guanosines (for example 7-deazaguanosine, which cannot form Hoogsteen base pairs) may be used to overcome this problem.

ONs containing CpG motifs should be excluded for *in vivo* experiments, because this motif is known to stimulate immune responses in mammalian systems. The CG dinucleotide is more frequently found in viral and bacterial DNA than in the human genome, suggesting that it is a marker for the immune system to signify infection. Coley Pharmaceuticals even makes use of CG-containing ONs as immune stimulants for treating cancer, asthma and infectious diseases in clinical trials [16].

Another important step for the development of an antisense molecule is to perform a database search for each ON sequence to avoid significant homology with other mRNAs. Furthermore, control experiments should be carried out with great care in order to prove that any observed effect is due to a specific antisense knockdown of the target mRNA. A number of types of control ONs have been used for antisense experiments: random ONs, scrambled ONs with the same nucleotide composition as the AS-ON in random order, sense ONs, ONs with the inverted sequence or mismatch ONs, which differ from the AS-ON in a few positions only.

In the following sections, properties of modified AS-ONs and recent advances obtained with novel DNA and RNA analogs will be discussed in more detail. Subsequently, strategies to mediate efficient cellular uptake of oligonucleotides and results of clinical trials will be described.

Antisense-oligonucleotide modifications

One of the major challenges for antisense approaches is the stabilization of ONs, as unmodified oligodeoxynucleotides are rapidly degraded in biological fluids by nucleases. A vast number of chemically modified nucleotides have been used in antisense experiments. In general, three types of modifications of ribonucleotides can be distinguished (Fig. 3): analogs with unnatural bases, modified sugars (especially at the 2′ position of the ribose) or altered phosphate backbones.

A variety of heterocyclic modifications have been described, which can be introduced into AS-ONs to strengthen base-pairing and thus stabilize the duplex between AS-ONs and their target mRNAs. A comprehensive review dealing with base-modified ONs was published previously by Herdewijn [17]. Because only a relatively small number of these ONs have been investigated *in vivo*, little is known about their potential as antisense molecules and their possible toxic side-effects. Therefore, the present review will focus on ONs with modified sugar moieties and phosphate backbones.

'First generation' antisense-oligonucleotides

Phosphorothioate (PS) oligodeoxynucleotides are the major representatives of first generation DNA analogs that are the best known and most widely used AS-ONs to date (reviewed in [18]). In this class of ONs, one of the nonbridging oxygen atoms in the phophodiester bond is replaced by sulfur (Fig. 4). PS DNA ONs were first synthesized in the 1960s by Eckstein and colleagues [19] and were first used as AS-ONs for the inhibition of HIV

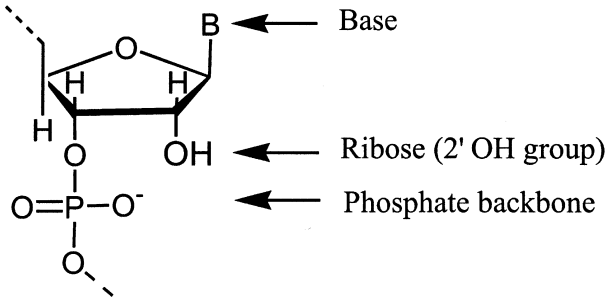

Fig. 3. Sites for chemical modifications of ribonucleotides. B denotes one of the bases adenine, guanine, cytosine or thymine.

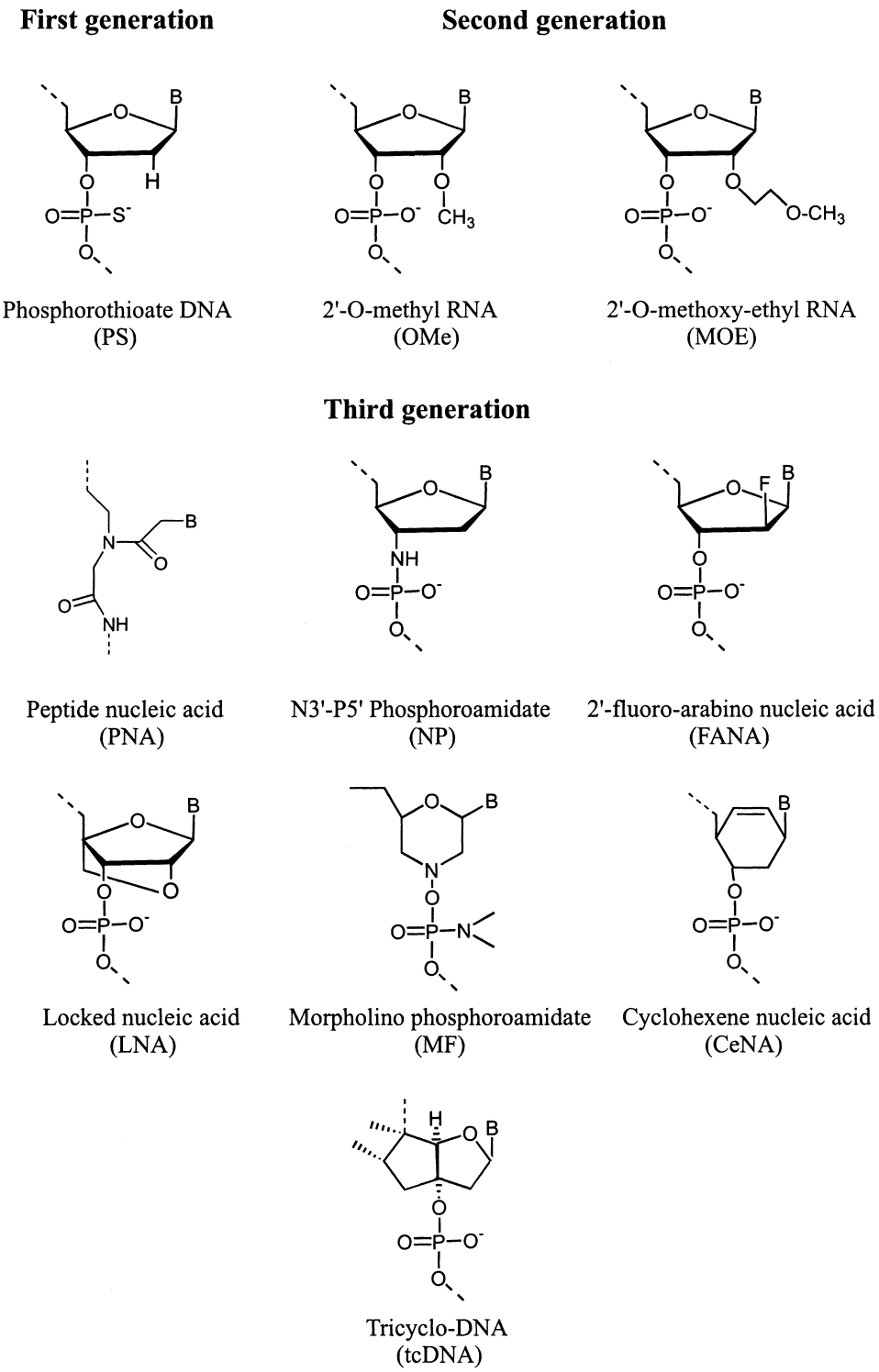

Fig. 4. Nucleic acid analogs discussed in this review. B denotes one of the bases adenine, guanine, cytosine or thymine.

replication by Matsukura and coworkers [20]. As described below, these ONs combine several desired properties for antisense experiments, but they also possess undesirable features.

The introduction of phosphorothioate linkages into ONs was primarily intended to enhance their nuclease resistance. PS DNAs have a half-life in human serum of approximately 9–10 h compared to $\approx$1 h for unmodified oligodeoxynucleotides [21–23]. In addition to nuclease resistance, PS DNAs form regular Watson–Crick base pairs, activate RNase H, carry negative charges for cell delivery and display attractive pharmacokinetic properties [24].

The major disadvantage of PS oligodeoxynucleotides is their binding to certain proteins, particularly those that interact with polyanions such as heparin-binding proteins (e.g. [25–27]). The reason for this nonspecific interaction is not yet fully understood, but it may cause cellular toxicity [reviewed in 28]. After PS DNA treatment of primates, serious acute toxicity was observed as a result of a transient activation of the complement cascade that has in some cases led to cardiovascular collapse and death. In addition, the clotting cascade was altered after the administration of PS DNA ONs. The lower doses of PS oligodeoxynucleotide used for clinical trials in humans, however, were generally well tolerated, as will be discussed below. Furthermore, the seemingly negative property of PS DNA ONs to interact with certain proteins proved to be advantageous for the pharmacokinetic profile. Their binding to plasma proteins protects them from filtration and is responsible for an increased serum half-life [28].

Another shortcoming of PS DNAs is their slightly reduced affinity towards complementary RNA molecules in comparison to their corresponding phosphodiester oligodeoxynucleotide. The melting temperature of a heteroduplex is decreased by approximately 0.5 °C per nucleotide. This weakness is, in part, compensated by an enhanced specificity of hybridization found for PS ONs compared to unmodified DNA ONs [24].

'Second generation' antisense-oligonucleotides

The problems associated with phosphorothioate oligodeoxynucleotides are to some degree solved in second generation ONs containing nucleotides with alkyl modifications at the 2′ position of the ribose. 2′-*O*-methyl and 2′-*O*-methoxy-ethyl RNA (Fig. 4) are the most important members of this class. AS-ONs made of these building blocks are less toxic than phosphorothioate DNAs and have a slightly enhanced affinity towards their complementary RNAs [23,29].

These desirable properties are, however, counterbalanced by the fact that 2′-*O*-alkyl RNA cannot induce RNase H cleavage of the target RNA. Mechanistic studies of the RNase H reaction revealed that the correct width of the minor groove of the AS-ON·RNA duplex (closer to A-type rather than B-type), flexibility of the AS-ON and availability of the 2′-OH group of the RNA are required for efficient RNase H cleavage [30].

Because 2′-*O*-alkyl RNA ONs do not recruit RNase H, their antisense effect can only be due to a steric block of translation (see above). The effectiveness of this mechanism was first shown in 1997, when the expression of the intercellular adhesion molecule 1 (ICAM-1) could be inhibited efficiently with an RNase H-independent 2′-*O*-methoxy-ethyl-modified AS-ON that was targeted against the 5′-cap region [31]. This effect was probably due to selective interference with the formation of the 80S translation initiation complex.

Another approach, for which the ON must avoid activation of RNase H, is an alteration of splicing. In contrast to the typical role for AS-ONs, in which they are supposed to suppress protein expression, blocking of a splice site with an AS-ON can increase the expression of an alternatively spliced protein variant. This technique is being developed to treat the genetic blood disorder β-thalassemia. In one form of this disease, a mutation in intron 2 of the β-globin gene causes aberrant splicing of the pre-mRNA and, as a consequence, β-globin deficiency. A phosphorothioate 2′-*O*-methyl oligoribonucleotide that does not induce RNase H cleavage was targeted to the aberrant splice site and restored correct splicing, generating correct β-globin mRNA and protein in mammalian cells [32].

For most antisense approaches, however, target RNA cleavage by RNase H is desired in order to increase antisense potency. Therefore, 'gapmer technology' has been developed. Gapmers consist of a central stretch of DNA or phosphorothioate DNA monomers and modified nucleotides such as 2′-*O*-methyl RNA at each end (indicated by red and yellow regions of the ON in Fig. 2B). The end blocks prevent nucleolytic degradation of the AS-ON and the contiguous stretch of at least four or five deoxy residues between flanking 2′-*O*-methyl nucleotides was reported to be sufficient for activation of *Escherichia coli* and human RNase H, respectively [29,33,34].

The use of gapmers has also been suggested as a solution for another problem associated with AS-ONs, the so-called 'irrelevant cleavage' [5]. The specificity of an AS-ON is reduced by the fact that it nests a number of shorter sequences. A 15-mer, for example, can be viewed as eight overlapping 8-mers, which are sufficient to activate RNase H. Each of these 8-mers will occur several times in the genome and might bind to nontargeted mRNAs and induce their cleavage by RNase H. This theoretical calculation became relevant for a 20-mer phosphorothioate oligodeoxyribonucleotide targeting the 3′-untranslated region of PKC-α. Unexpectedly, PKC-ζ was codown-regulated by the ON, probably due to irrelevant cleavage caused by a contiguous 11-base match between the ON and the PKC-ζ mRNA. Gapmers with a central core of six to eight oligodeoxynucleotides and nucleotides unable to recruit RNase H at both ends can be employed to eliminate irrelevant cleavage, as they will only induce RNase H cleavage of one target sequence.

'Third generation' antisense-oligonucleotides

In recent years a variety of modified nucleotides have been developed (Fig. 4) to improve properties such as target affinity, nuclease resistance and pharmacokinetics. The concept of conformational restriction has been used widely to enhance binding affinity and biostability. In analogy to the previous terms 'first generation' for phosphorothioate DNA and 'second generation' for 2′-*O*-alkyl-RNA, these novel nucleotides will subsequently be subsumed under the term 'third generation' antisense agents. DNA and RNA analogs with modified phosphate linkages or riboses as well as nucleotides with a completely different chemical moiety substituting the furanose ring have been developed, as will be described below. Due to the limited space, only a few promising examples of the vast body of novel modified nucleotides with improved properties can be discussed here, although further modifications may prove to have a great potential as antisense molecules.

Peptide nucleic acids (PNAs). Peptide nucleic acids (PNAs) belong to the first and most intensively studied DNA analogs besides phosphorothioate DNA and 2′-*O*-alkyl RNA [reviewed in 35–37]. In PNAs the deoxyribose phosphate backbone is replaced by polyamide linkages. PNA was first introduced by Nielsen and coworkers in 1991 [38] and can now be obtained commercially, e.g. from Applied Biosystems (Foster City, CA, USA). PNAs have favorable hybridization properties and high biological stability, but do not elicit target RNA cleavage by RNase H. Additionally, as they are electrostatically neutral molecules, solubility and cellular uptake are serious problems that have to be overcome for the usage of PNAs as antisense agents to become practical. Improved intracellular delivery could be obtained by coupling PNAs to negatively charged oligomers, lipids or certain peptides that are efficiently internalized by cells [summarized in 35,37].

In one of the latest and most convincing *in vivo* studies, PNAs (as well as several other modified ONs) were used to correct aberrant splicing in a transgenic mouse model [39]. The ONs were directed against a mutated intron of the human β-globin gene that interrupted the gene encoding enhanced green fluorescent protein (GFP). Only in the presence of systemically delivered AS-ONs was the functional GFP expressed. Interestingly, PNAs linked to four lysines at the C-terminus were the most effective of the AS-ONs investigated, whereas a 2′-*O*-methoxy-ethyl ON had a slightly lower activity in all tissues except the small intestine. Morpholino (MF) ONs were significantly less effective while PNA with only one lysine was completely inactive, indicating that the four-lysine tail is essential for antisense activity of PNAs *in vivo*.

According to the *in vivo* studies performed to date, PNAs seem to be nontoxic, as they are uncharged molecules with low affinity for proteins that normally bind nucleic acids. The greatest potential of PNAs, however, might not be their use as antisense agents but their application to modulate gene expression by strand invasion of chromosomal duplex DNA [37].

N3′-P5′ phosphoroamidates (NPs). N3′-P5′ phosphoroamidates (NPs) are another example of a modified phosphate backbone, in which the 3′-hydroxyl group of the 2′-deoxyribose ring is replaced by a 3′-amino group. NPs exhibit both a high affinity towards a complementary RNA strand and nuclease resistance [40]. Their potency as AS molecules has already been demonstrated *in vivo*, where a phosphoroamidate ON was used to specifically down-regulate the expression of the *c-myc* gene [41]. As a consequence, severe combined immunodeficiency mice that were injected with myeloid leukemia cells had a reduced peripheral blood leukemic load. Animals treated with the AS agent had significantly prolonged survival compared to those treated with mismatch ONs. Moreover, the phosphoroamidates were found to be superior for the treatment of leukemia compared to phosphorothioate oligodeoxynucleotides. The sequence specificity of phosphoroamidate-mediated antisense effects by steric blocking of translation initiation could be demonstrated in cell culture, and *in vivo* with a system in which the target sequence was present just upstream of the firefly luciferase initiation codon [42]. Because phosphoroamidates do not induce RNase H cleavage of the target RNA, they might prove useful for special applications, where RNA integrity needs to be maintained, like modulation of splicing.

*2′-Deoxy-2′-fluoro-β-*D*-arabino nucleic acid (FANA).* ONs made of arabino nucleic acid, the 2′ epimer of RNA, or the corresponding 2′-deoxy-2′-fluoro-β-D-arabino nucleic acid analogue (FANA) were the first uniformly sugar-modified AS-ONs reported to induce RNase H cleavage of a bound RNA molecule [43]. The circular dichroic spectrum of a FANA·RNA duplex closely resembled that of the corresponding DNA·RNA hybrid, indicating similar helical conformations. The fluoro substituent is thought to project into the major groove of the helix, where it should not interfere with RNase H. Full RNase H activation by phosphorothioate–FANA, however, was only achieved with chimeric ONs containing deoxyribonucleotides in the center, but the DNA stretch needed for high enzyme activity was shorter than in 2′-*O*-methyl gapmers [44]. The chimeric FANA·DNA ONs were highly potent in cell culture with a 30-fold lower IC_{50} than the corresponding phosphorothioate DNA ON.

Locked nucleic acid (LNA). One of the most promising candidates of chemically modified nucleotides developed in the last few years is locked nucleic acid (LNA), a ribonucleotide containing a methylene bridge that connects the 2′-oxygen of the ribose with the 4′-carbon [reviewed in 36,45,46]. ONs containing LNA were first synthesized in the Wengel [47,48] and Imanishi laboratories [49] and are commercially available from Proligo (Paris, France and Boulder, CO, USA).

Introduction of LNA into a DNA ON induces a conformational change of the DNA·RNA duplex towards the A-type helix [50] and therefore prevents RNase H cleavage of the target RNA. If degradation of the mRNA is intended, a chimeric DNA·LNA gapmer that contains a stretch of 7–8 DNA monomers in the center to induce RNase H activity should be used [23]. Chimeric 2′-*O*-methyl–LNA ONs that do not activate RNase H could, however, be used as steric blocks to inhibit intracellular HIV-1 Tat-dependent *trans* activation and hence suppress gene expression [51]. LNAs and LNA·DNA chimeras efficiently inhibited gene expression when targeted to a variety of regions (5′ untranslated region, region of the start codon or coding region) within the luciferase mRNA [52].

Chimeric DNA·LNA ONs reveal an enhanced stability against nucleolytic degradation [23,53] and an extraordinarily high target affinity. An increase of the melting temperature of up to 9.6 °C per LNA introduced into an ON has been reported [50]. This enhanced affinity towards the target RNA accelerates RNase H cleavage [23] and leads to a much higher potency of chimeric DNA·LNA ONs in suppressing gene expression in cell culture, compared to phosphorothioate DNAs or 2′-*O*-methyl modified gapmers [A. Grünweller, E. Wyszko, V. A. Erdmann and J. Kurreck, unpublished results]. Whether the high target affinity of LNAs results in a reduced sequence specificity will need to be investigated. If unspecific side-effects of LNA

ONs are observed, their length would have to be decreased to find an optimum for target affinity and specificity.

AS-ONs containing LNA were also directed against human telomerase, which is an excellent antisense target that is expressed in tumor cells but not in adjacent normal tissue. Telomerase is a ribonucleoprotein with an RNA component that hybridizes to the telomere and should therefore be accessible for AS-ONs. As RNA degradation is not necessary to block the enzyme's catalytic site, ONs unable to recruit RNase H should be suitable inhibitors of telomerase function. A comparative study revealed that LNAs have a significantly higher potential to inhibit human telomerase than PNAs [54]. Due to their high affinity for their complementary sequence, LNA ONs as short as eight nucleotides long were efficient inhibitors in cell extracts.

In addition to target affinity, improved cellular uptake of ONs consisting of 2′-*O*-methyl RNA and LNA, compared to an all 2′-*O*-methyl RNA oligomer, was suggested to account for high antisense potency of LNA [51]. In the first *in vivo* study reported for an LNA, an efficient knock-down of the rat delta opioid receptor was achieved in the absence of any detectable toxic reactions in rat brain [53]. Subsequently, full LNA ONs were successfully used *in vivo* to block the translation of the large subunit of RNA polymerase II [55]. These ONs inhibited tumor growth in a xenograft model with an effective concentration that was five times lower than was found previously for the corresponding phosphorothioate DNA. Again, the LNA ONs appeared to be nontoxic in the optimal dosage. Therefore, full LNA and chimeric DNA·LNA ONs seem to offer an attractive set of properties, such as stability against nucleolytic degradation, high target affinity, potent biological activity and apparent lack of acute toxicity.

Morpholino oligonucleotides (MF). Morpholino ONs are nonionic DNA analogs, in which the ribose is replaced by a morpholino moiety and phosphoroamidate intersubunit linkages are used instead of phosphodiester bonds. They are commercially available from Gene Tools LLC (Corvallis, OR, USA). Recently, the success and limitations of their usage have been reviewed comprehensively, with particular focus on developmental biology [56] as most published work on morpholino compounds has been carried out using zebrafish embryos. An entire issue of *Genesis* (volume 30, issue 3, 2001) has been devoted to the study of gene function using this technique.

MFs do not activate RNase H and, if inhibition of gene expression is desired, they should therefore be targeted to the 5′ untranslated region or to the first 25 bases downstream of the start codon to block translation by preventing ribosomes from binding. Because their backbone is uncharged, MFs are unlikely to form unwanted interactions with nucleic acid-binding proteins. Their target affinity is similar to that of isosequential DNA ONs, but lower than the strength of RNA binding achieved with many of the other modifications described in this section.

Effective gene knockdown in all cells of zebrafish embryos was achieved with MFs against GFP in a ubiquitous GFP transgene [57]. In this study, equivalents of known genetic mutants as well as models for human diseases were developed and new gene functions were determined by the use of MFs. A potential therapeutic application was reported for MFs that corrected aberrant splicing of mutant β-globin precursor mRNA [58]. Treatment of erythroid progenitors from peripheral blood of thalassemic patients with ONs antisense to aberrant splice sites restored correct splicing and increased the hemoglobin A synthesis. Due to the limited cellular uptake of MFs, however, these experiments required high ON concentrations and mechanical disturbance of the cell membrane. Another relevant question that has to be answered is the reason for unspecific side-effects that have been observed in several studies (summarized in [56]).

Cyclohexene nucleic acids (CeNA). Replacement of the five-membered furanose ring by a six-membered ring is the basis for cyclohexene nucleic acids (CeNAs), which are characterized by a high degree of conformational rigidity of the oligomers. They form stable duplexes with complementary DNA or RNA and protect ONs against nucleolytic degradation [59]. In addition, CeNA·RNA hybrids have been reported to activate RNase H, albeit with a 600-fold lower k_{cat} compared to a DNA·RNA duplex [60]. Therefore, the design of ONs with CeNA has a long way to go in order to obtain highly efficient AS agents.

Tricyclo-DNA (tcDNA). Tricyclo-DNA (tcDNA) is another nucleotide with enhanced binding to complementary sequences, which was first synthesized by Leumann and coworkers [61,62]. As with most of the newly developed DNA and RNA analogs, tcDNA does not activate RNase H cleavage of the target mRNA. It was, however, successfully used to correct aberrant splicing of a mutated β-globin mRNA with a 100-fold enhanced efficiency relative to an isosequential 2′-*O*-methyl-phosphorothioate RNA [63].

In summary, a great number of modified building blocks for ONs have been developed during the last few years. Although not all of them could be discussed in the present review, general features have been shown for some promising examples. Most of the newly synthesized nucleotides reveal enhanced resistance against nucleolytic degradation in biological fluids and stabilize the duplex between the AS-ON and the mRNA. A major inherent disadvantage of nucleotides with modifications in the ribose moiety is their inability to activate efficient RNase H cleavage of the target RNA. As a consequence, gapmers with a stretch of unmodified or phosphorothioate DNA monomers in the center of the ON are widely used. Several of the third generation nucleotides have already been used successfully *in vivo*, and a high antisense potency combined with low toxicity has been observed. Therefore, one might expect that recent advances in nucleotide chemistry will soon lead to significant improvements of the antisense technology for target validation and therapeutic purposes.

Cellular uptake of antisense-oligonucleotides

Despite the encouraging prospects of nucleotide chemistry discussed in the previous section, an important hurdle that has to be overcome for successful antisense applications is the cellular uptake of the molecules. In cultured cells, internalization of naked DNA is usually inefficient, due to the charged ONs having to cross a hydrophobic cell

membrane. A number of methods have therefore been developed for *in vitro* and *in vivo* delivery of ONs (reviewed in [64,65]). By far the most commonly and successfully used delivery systems are liposomes and charged lipids, which can either encapsulate nucleic acids within their aqueous center or form lipid–nucleic acid complexes as a result of opposing charges. These complexes are usually internalized by endocytosis. For efficient release of the ONs from the endosomal compartment, many transfection reagents contain helper lipids that disrupt the endosomal membrane and help to set the ONs free.

A number of macromolar delivery systems have been developed recently that mediate a highly efficient cellular uptake and protect the bound ONs against degradation in biological fluids. Examples of these new agents are dendrimers with highly branched three dimensional structures, biodegradable polymers and ON-binding nanoparticles. In addition, pluoronic gel as a depot reservoir can be used to deliver ONs over a prolonged period [66]. It has been used *in vivo* successfully for the delivery of DNA enzymes (see below), which inhibited neointima formation after balloon injury to the rat carotid wall [67,68].

Further polymers for the delivery of AS-ONs consist of amino acids or sugars. Evidence has been provided, however, that the structural properties of a peptide conjugated to an ON do not significantly alter its ability to cross mammalian plasma membranes [69]. Therefore, aspects other than improved translocation across the membrane are likely to be responsible for enhanced biological activity of peptide–oligonucleotide derivatives. Further details about the newly developed delivery systems and perspectives for their wider use are given in the reviews mentioned above [64,65].

Another strategy for effective targeting of AS-ONs to specific tissues or organs is receptor-mediated endocytosis. For this purpose, ONs are conjugated to antibodies or ligands that are specifically recognized by a certain receptor, which mediates their uptake into target cells. For example, coupling of a radioactively labeled PNA to a transferrin receptor monoclonal antibody made the antisense agent transportable through the blood–brain barrier [70].

Interestingly, efficient cellular uptake of ONs *in vivo* has even been achieved without the use of any delivery system. In a recently published study it was demonstrated that fluorescently labeled AS-ONs were taken up by dorsal root ganglion neurons after intrathecal injection in the absence of any transfection agent [71]. The ONs specifically knocked down the expression of the peripheral tetrodoxin-resistant sodium channel NaV1.8 and reversed neuropathic pain induced by spinal nerve injury. Internalization into target cells *in vivo* has also been achieved for free ribozymes (see below). Despite these successful applications of free antisense molecules, higher levels of cellular uptake can usually be achieved by the use of transfection agents. Therefore, the development of delivery systems that mediate efficient cellular uptake and sustained release of the drugs remains one of the major challenges in the antisense field.

Clinical trials

After pharmacokinetic studies had shown that phosphorothioate oligodeoxynucleotides are well absorbed from parenteral sites and distribute broadly to organs and peripheral tissues [24] (with the exception that they do not cross the blood–brain barrier in the absence of special delivery systems) several companies initiated clinical trials in the early 1990s. As can be seen from the summary of ongoing clinical studies given in Table 1, the most intensively studied AS-ONs are phosphorothioate DNA ONs, but second and third generation ONs have meanwhile proceeded to Phase II trials. The list also demonstrates the

Table 1. Antisense-oligonucleotides approved or in clinical trials (compilation based on 16,37,81 and company's information).

Product	Company	Target	Disease	Chemistry	Status
Vitravene (Fomivirsen)	ISIS Pharmaceuticals	CMV IE2	CMV retinitis	PS DNA	Approved
Affinitac (ISIS 3521)	ISIS	PKC-α	Cancer	PS DNA	Phase III
Genasense	Genta	Bcl2	Cancer	PS DNA	Phase III
Alicaforsen (ISIS 2302)	ISIS	ICAM-1	Psoriasis, Crohn's disease, Ulcerative colitis	PS DNA	Phase II/III
ISIS 14803	ISIS	Antiviral	Hepatitis C	PS DNA	Phase II
ISIS 2503	ISIS	H-ras	Cancer	PS DNA	Phase II
MG98	Methylgene	DNA methyl transferase	Solid tumors	PS DNA	Phase II
EPI-2010	EpiGenesis Pharmaceuticals	Adenosine A1 receptor	Asthma	PS DNA	Phase II
GTI 2040	Lorus Therapeutics	Ribonucleotide reductase (R2)	Cancer	PS DNA	Phase II
ISIS 104838	ISIS	TNF α	Rheumatoid Arthritis, Psoriasis	2nd generation	Phase II
Avi4126	AVI BioPharma	c-myc	Restenosis, cancer, Polycystic kidney disease	3rd generation	Phase I/II
Gem231	Hybridon	PKA RIα	Solid tumors	2nd generation	Phase I/II
Gem92	Hybridon	HIV gag	AIDS	2nd generation	Phase I
GTI 2051	Lorus Therapeutics	Ribonucleotide reductase (R1)	Cancer	PS DNA	Phase I
Avi4557	AVI BioPharma	CYP3A4	Metabolic redirection of approved drugs	3rd generation	Phase I

almost universal applicability of antisense strategies to treat a broad range of diseases including viral infections, cancer and inflammatory diseases.

In 1998, the first (and to date only) antisense drug Vitravene (Fomivirsen), was approved by the US Food and Drug Administration [72]. The phosphorothioate DNA is intravitreally injected to treat cytomegalovirus-induced retinitis in patients with AIDS. Approval of Vitravene was a milestone for companies involved in the antisense field. The drug meets an important need for affected patients, but its application is rare so that it generated only about $157 000 in sales for ISIS Pharmaceuticals (Carlsbad, CA, USA) and Novartis (Basel, Switzerland) in 2001 [16].

Three antisense phosphorothioate oligodeoxynucleotides are currently being investigated in Phase III trials. Affinitac (ISIS 3521) is targeted against the protein kinase C-alpha (PKC-α) for the treatment of nonsmall-cell lung cancer. The successful trial caught the attention of big pharmaceutical companies and led to a $200 million deal between Eli Lilly (Indianapolis, IN, USA) and ISIS Pharmaceuticals [73]. This deal marked the recovery from a serious setback for ISIS in 1999, when Alicaforsen (ISIS 2302) failed to show significant efficacy in a Phase III study, where it was tested for treatment of Crohn's disease [74]. This AS-ON is now being investigated in a restructured Phase III trial. Genta (Berkeley Hights, NJ, USA) is developing the anticancer drug Genasense, which attacks the apoptosis inhibitor Bcl2 and shows antitumor responses in patients with malignant melanomas [75].

Further antiviral or anticancer phosphorothioate DNAs are being investigated in Phase I or II trials. Most of the antisense molecules currently being tested are intravenously or subcutaneously injected, but EpiGenesis Pharmaceuticals (Cranbury, NJ, USA) developed a 'respirable antisense-oligonucleotide' (RASON) targeting the adenosine A_1 receptor to treat asthma [76]. It has a duration of effect of approximately one week, giving it the potential to be the first once-per-week treatment for this disease.

Recently, results of a pilot study for the treatment of chronic myelogenous leukemia patients were presented [77]. Marrow cells were purged *ex vivo* with a phosphorothioate oligodeoxynucleotide against the short-lived *c-myb* proto-oncogene. The treatment led to major cytogenetic remissions in six of an evaluable 14 patients. An infusion trial with the c-myb AS-ONs in patients with refractory leukemia of all types has been approved and is expected be started soon (A. M. Gewirtz, Division of Haematology/Oncology, University of Pennsylvania School of Medicine, Philadelphia, USA, personal communication).

Furthermore, several second generation ONs have reached the stage of clinical trials. ISIS 104838 against tumor necrosis factor α (TNFα) is being tested for the treatment of inflammatory diseases such as rheumatoid arthritis and psoriasis, and Hybridon (Cambridge, MA, USA) uses second generation drug candidates to treat cancer and HIV infections. Mixed backbone oligonucleotides consisting of phosphorothioate internucleotide linkages and four 2′-*O*-methyl RNA nucleotides at both ends were shown to have antitumor activity in mice after oral administration [78].

Mixed backbone oligonucleotides usually contain phosphorothioate internucleotide linkages even between the 2′-*O*-methyl nucleotides. Thus, the number of phosphorothioates is not decreased compared to an entirely phosphorothioate DNA ON, but for reasons unknown to date their toxicity is significantly reduced. Regardless of this open question, AS-ONs containing second generation modifications combine several advantageous properties, including higher *in vivo* stability, better pharmacological and toxicological profiles and the opportunity for oral administration.

Third generation AS-ONs with a morpholino-type backbone are being tested in Phase I and II clinical trials by Avi BioPharma (Portland, OR, USA). Avi4126 targets the oncogene *c-myc* and is used to treat restenosis, polycystic kidney disease and solid tumors [79]. A second MF-ON against cytochrome P450 (CYP3A4) is being designed for metabolic redirection of approved drugs. An N3′-P5′-thiophosphoroamidate that efficiently inhibited telomerase activity in spontaneously immortalized human breast epithelial cells [80] will soon be moved to clinical trials by Geron (Menlo Park, CA; S. Gryaznov, personal communication).

Although the AS molecules have been well-tolerated in most cases and some results were encouraging, no or only minor responses were achieved in several studies [81]. Taken together, an increasing number of AS-ONs have been investigated in different stages of clinical trials and a broad spectrum of diseases is addressed in these studies, but some questions remain to be answered. Solutions to major problems of serum-stability, bioavailability, tissue-targeting and cellular delivery urgently need to be found. Most of the antisense molecules used are still phosphorothioate oligodeoxynucleotides, but some second and third generation chemistry molecules are being tested and seem to provide favorable pharmacokinetic properties and the opportunity of oral administration.

Ribozymes

In the early 1980s, Cech and coworkers discovered the self-splicing activity of the group I intron of *Tetrahymena thermophilia* [82,83] and coined the term 'ribozymes' to describe these RNA enzymes. Shortly thereafter, Altman and colleagues discovered the active role of the RNA component of RNase P in the process of tRNA maturation [84]. This was the first characterization of a true RNA enzyme that catalyses the reaction of a free substrate, i.e. possesses catalytic activity *in trans*. A variety of ribozymes, catalyzing intramolecular splicing or cleavage reactions, have subsequently been found in lower eukaryotes, viruses and some bacteria. The different types of ribozymes and their mechanisms of action have been described comprehensively [85–89] and the present review will therefore focus on the stabilization and medical application of the hammerhead ribozyme, which has been studied in great detail and is one of the most widely used catalytic RNA molecules.

The hammerhead ribozyme was isolated from viroid RNA and its dissection into enzyme and substrate strands [90,91] transformed this *cis*-cleaving molecule into a target-specific *trans*-cleaving enzyme with a great potential for applications in biological systems. This minimized hammerhead ribozyme is less than 40 nucleotides long and consists of two substrate binding arms and a catalytic domain (Fig. 5).

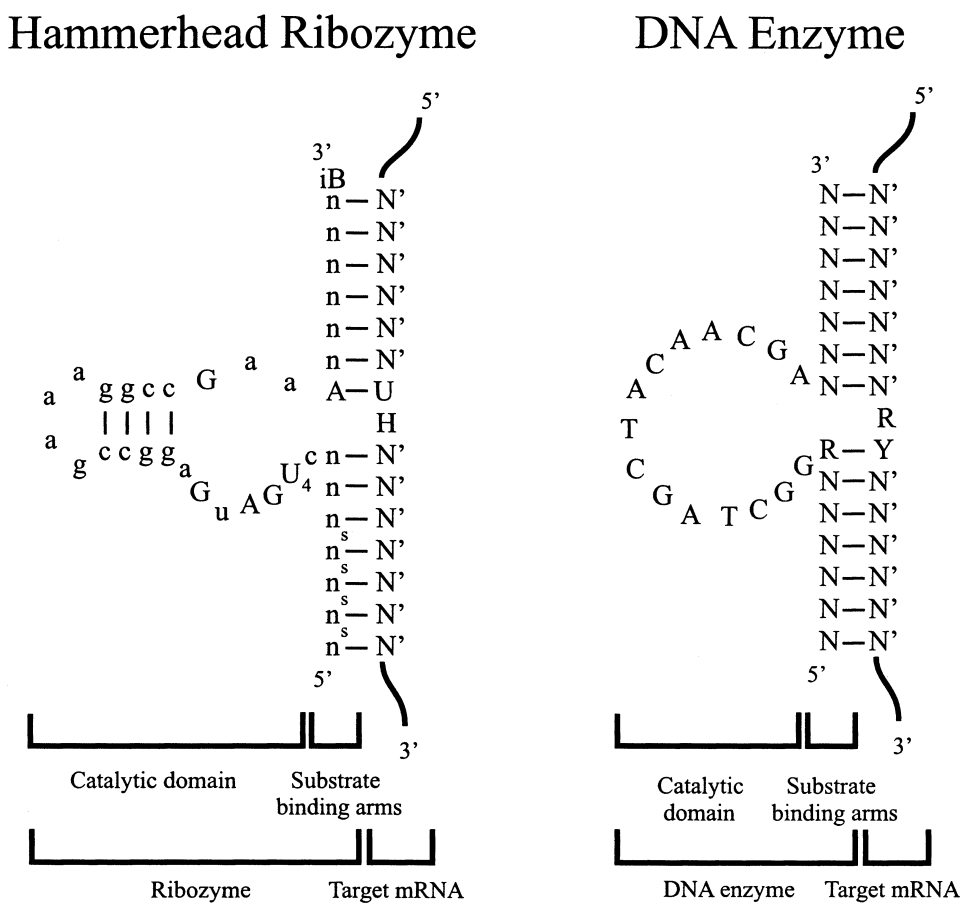

Fig. 5. Secondary structure models for the hammerhead ribozyme and the 10-23 DNA enzyme. A nuclease-resistant ribozyme according to Usman and Blatt [111] is shown. It consists of 2′-*O*-methyl RNA (lower case), five ribonucleotides (upper case), a 2′-C-allyluridin at position 4, four phosphorothioate linkages (s) and an inverted 3′-3′ deoxabasic sugar. The DNA enzyme shown consists entirely of DNA nucleotides; R is a purine, Y is a pyrimidine.

For the development of a therapeutic hammerhead ribozyme similar problems have to be solved as described for AS-ONs. Some steps, however, are more challenging due to the catalytic nature of ribozymes. Firstly, suitable target sites have to be identified, secondly the oligoribonucleotides have to be stabilized against nucleolytic degradation and thirdly the ribozymes have to be delivered into the target cells.

Hammerhead ribozymes are known to cleave any NUH triplets (where H is any nucleotide except guanosine) with AUC and GUC triplets being processed most efficiently. Triplets with a cytidine or an adenosine at the second position were reported to be cleavable by hammerhead ribozymes [92], although these reactions occurred at lower rates. Due to secondary and tertiary structures of the target mRNAs, not all sequences that are theoretically cleavable by hammerhead ribozymes are suitable for practical applications. Therefore, several assays have been developed to identify accessible target sites.

A good correlation was found for regions of the c-myb mRNA that were accessible to AS-ON binding in an RNase H assay and their susceptibility to cleavage by ribozymes *in vitro* [93]. Oligonucleotide scanning of the DNA methyltransferase mRNA in cell extracts had also been found to be predictive for ribozyme activity in cell extracts and inside cells [94].

Another approach for the identification of active ribozymes was based on the usage of libraries with randomized substrate recognition arms. The hammerhead ribozymes have either been transcribed from expression cassettes [95] or were chemically synthesized [96]. A highly sophisticated method was developed, in which a sequence-specific library of hammerhead ribozymes was generated by partial digestion of the target cDNA and subsequent introduction of the catalytic domain into the library [97].

For applications in cell culture or *in vivo*, ribozymes can either be transcribed from plasmids inside the target cells or they can be administered exogenously. The first approach requires the design of expression cassettes with an RNA polymerase III promoter and stem-loop structures that stabilize the ribozyme (reviewed in [98]). Some gene therapy-based trials have been performed to treat individuals infected with HIV (summarized in [99]). Because the use of chemically synthesized ribozymes proved to be more straightforward, this approach will be discussed in more detail below. Due to the fact that RNA is rapidly degraded in biological systems, presynthesized ribozymes have to be protected against nucleolytic attack before they can be used in cell culture or *in vivo*.

Stabilization of ribozymes is even more difficult than protection of AS-ONs, as the introduction of modified

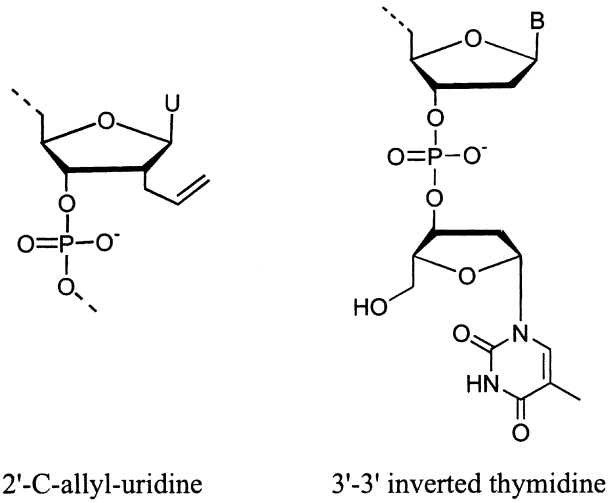

Fig. 6. Modified nucleotides used to stabilize ribozymes and DNA enzymes.

nucleotides very often leads to conformational changes that abolish catalytic activity. Based on a number of reports, in which sequence–function relationships in the hammerhead ribozyme were analyzed, a comprehensive study was performed using a great variety of modified nucleotides that led to an optimized design for a stabilized hammerhead ribozyme, which is almost as active as its unmodified parent [100]. The nuclease resistant ribozyme contains five unmodified ribonucleotides, a 2′-*C*-allyl uridine (Fig. 6) at position 4 and 2′-*O*-methyl RNA at all remaining positions. In addition, the 3′ end was protected by an inverted thymidine. The serum half-life of the stabilized ribozyme is increased to more than 10 days compared to a less than 1 min half-life of the unmodified RNA ribozyme. A slightly improved version of this ribozyme with four phosphorothioate bonds in one substrate recognition arm and an inverted 3′-3′ deoxyabasic sugar led to the design presented in Fig. 5 that is now used for clinical trials (see below).

The development of *in vitro* selection techniques using combinatorial libraries opened the road to generate ribozymes with advantageous properties such as the accessibility of new target sites [101], high activity under physiological Mg^{2+} concentrations [102] and enhanced biostability (reviewed in [103]). A highly active ribozyme against a *K-ras* target sequence could be selected in the presence of 2′-fluoro and 2′-amino modified ribonucleotides [104]. The optimized ribozyme that was named Zinzyme has a relatively high catalytic activity at 1 mM Mg^{2+} and cleaves a new Y-G-H (where Y is C or U, and H is A, C or U) target sequence. Two unmodified guanosines and two 2′-amino nucleotides are essential for cleavage activity, 2′-*O*-methyl RNA could be introduced at all other positions. The arms are further stabilized by phosphorothioate linkages and an inverted 3′-3′ deoxyabasic sugar as described above. The Zinzyme has a half-life of > 100 h in human serum.

Ribonucleotides, which are highly susceptible to nucleases, could be avoided entirely by the selection of an RNA-cleaving DNA enzyme [105]. The most prominent deoxyribozyme, named ′10-23′, consists of a catalytic core of 15 nucleotides and two substrate recognition arms of 6–12 nucleotides on either arm (Fig. 5). It is highly sequence-specific and can cleave any junction between a purine and a pyrimidine (review [106]). A comparative study of hammerhead ribozymes and DNA enzymes targeting the same cleavage sites of a long mRNA revealed that no general conclusions can be drawn as to whether the hammerhead ribozyme or the DNA enzyme is more efficient, but the most active cleaver found in the study was a 10-23 DNA enzyme [11].

Addition of an inverted nucleotide at the 3′ end enhanced serum stability of the 10-23 DNA enzyme 10-fold (the half-life of the modified DNA enzyme was 20 h compared to less than 2 h for the unmodified deoxyribozyme) [107]. DNA enzymes with a 3′-3′ inverted thymidine have also been used in the first *in vivo* application and inhibited neointima formation after balloon injury [67]. Sequence requirements in the catalytic core of the 10-23 DNA enzyme were analyzed and revealed a higher degree of conservation at the borders than in between [108]. A DNA enzyme with optimized substrate recognition arms and a partially protected catalytic domain possessed not only increased nuclease resistance but also enhanced catalytic activity [S. Schubert and J. Kurreck, unpublished results].

For transfection of eukaryotic cells with ribozymes, similar strategies can be used as have been described above for AS-ONs. Again, cationic lipids are most commonly used for cell culture experiments, but successful application of ribozymes in an animal model was demonstrated in the absence of any delivery system [109]. Chemically stabilized ribozymes were taken up by cells in the synovial lining after intra-articular administration and reduced the interleukin 1α-induced stromelysin mRNA. Higher transfection efficiencies can, however, usually be achieved with delivery systems. In addition, it could be shown that low molecular mass poly(ethylenimine) not only mediates highly efficient cellular uptake of ribozymes but also stabilizes RNA against nucleolytic degradation [110]. Poly(ethylenimine)-complexed ribozymes consisting of unmodified RNA were stable in cell culture and *in vivo*, and reduced tumor growth in a mouse xenograft model.

One of the leading companies in the field, Ribozyme Pharmaceuticals (Boulder, CO, USA), performs clinical trials (Table 2) using stabilized hammerhead ribozymes [111] as well as Zinzymes. ANGIOZYME is a stabilized hammerhead ribozyme that is targeted against the vascular endothelial growth factor (VEGF) receptor. It is designed to reduce tumor growth by inhibition of the formation of new blood vessels (angiogenesis). An additional benefit is expected from the combination of ANGIOZYME with chemotherapy in the treatment of metastatic colorectal

Table 2. Chemically synthesized ribozymes of Ribozyme Pharmaceuticals in ongoing clinical trials (P. Pavco, Ribozyme Pharmaceuticals, personal communication).

Product	Target	Disease	Status
ANGIOZYME	VEGF-receptor 1	Metastatic colorectal cancer	Phase II
HERZYME	HER-2	Cancer	Phase I

cancer. For further details about the current status of ribozymes as therapeutic agents for cancer and problems in progressing from cell culture studies to *in vivo* models and clinical trials, see Wright and Kearney [112].

HEPTAZYME is another modified hammerhead ribozyme that cleaves the internal ribosome entry site of the Hepatitis C virus. The ribozyme was demonstrated to inhibit viral replication up to 90% in cell culture [113]. HEPTAZYME was tested in a Phase II study, but is no longer in a clinical trial (P. Pavco, Ribozyme Pharmaceuticals, personal communication). HERZYME is a Zinzyme that is targeted against the human epidermal growth factor-2 (HER2), which is overexpressed in certain breast and ovarian cancers. This ribozyme is being tested in a Phase I trial (P. Pavco, Ribozyme Pharmaceuticals, personal communication) to gain information about the safety and the adequateness of the pharmacokinetics of HERZYME.

RNA interference

Only recently, research in the antisense field increased in impact by the discovery of RNA interference (RNAi). This naturally occurring phenomenon as a potent sequence-specific mechanism for post-transcriptional gene silencing was first described for the nematode worm *Caenorhabditis elegans* [114]. Due to the advances made in the RNAi field during the last two years, numerous reviews have been published only recently [115–117]. RNA interference is initiated by long double-stranded RNA molecules, which

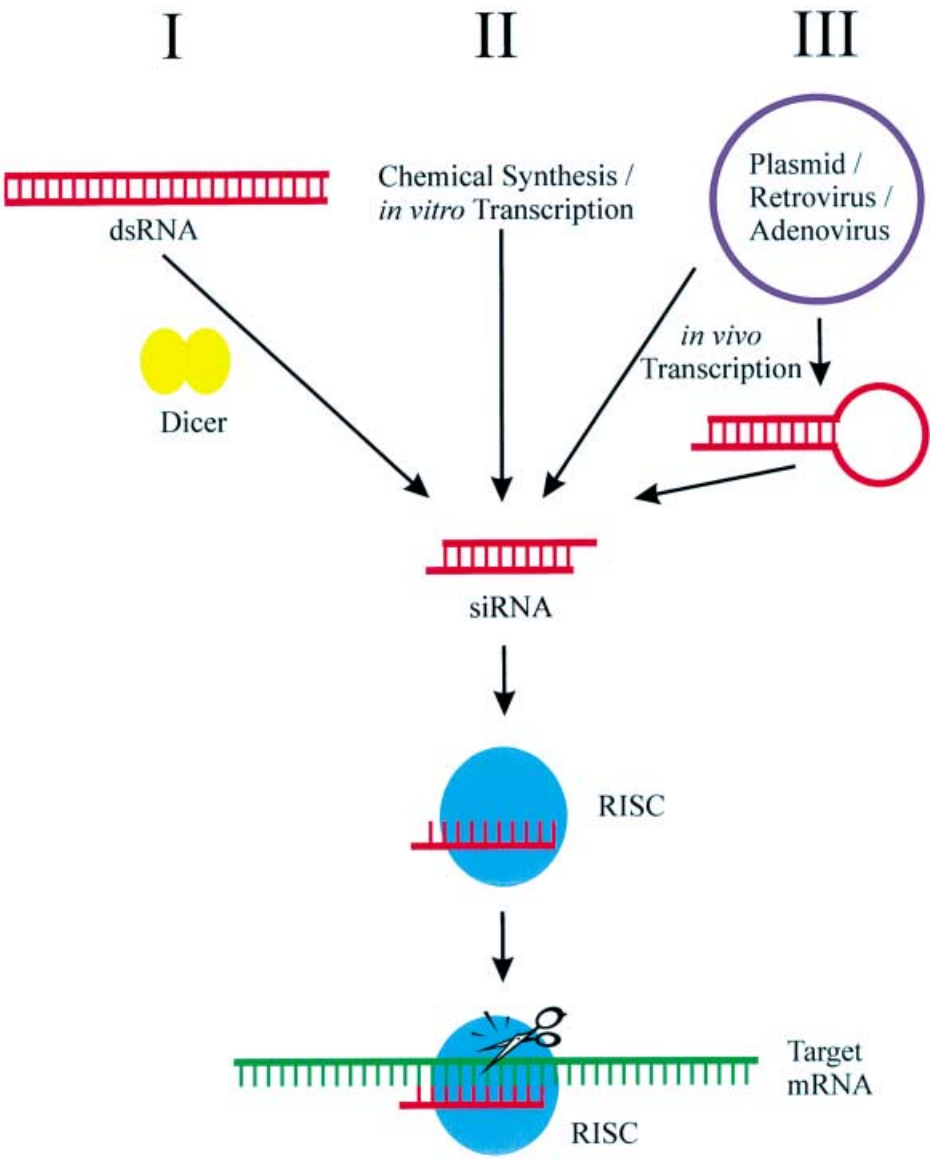

Fig. 7. Gene silencing by RNA interference (RNAi). RNAi is triggered by siRNAs, which can by generated in three ways. (I) Long double-stranded RNA molecules are processed into siRNA by the Dicer enzyme; (II) chemically synthesized or *in vitro* transcribed siRNA duplexes can be transfected into cells; (III) the siRNA molecules can be generated *in vivo* from plasmids, retroviral vectors or adenoviruses. The siRNA is incorporated into the RISC and guides a nuclease to the target RNA.

are processed into 21–23 nucleotides long RNAs by the Dicer enzyme (Fig. 7). This RNase III protein is thought to act as a dimer that cleaves both strands of dsRNAs and leaves two-nucleotide, 3′ overhanging ends. These small interfering RNAs (siRNAs) are then incorporated into the RNA-induced silencing complex (RISC), a protein·RNA complex, and guide a nuclease, which degrades the target RNA.

This conserved biochemical mechanism could be used to study gene functions in a variety of model organisms, but its application to mammalian cells was hampered by the fact that long double-stranded RNA molecules induce an interferon response. It was therefore a revolutionary breakthrough, when Tuschl and coworkers could show that 21 nucleotide-long siRNA duplexes with 3′ overhangs can specifically suppress gene expression in mammalian cells [2]. This finding triggered an enormous number of studies using RNAi in mammalian cells, as it is thought to provide a significantly higher potency compared to traditional antisense approaches.

Interestingly, not only short double-stranded RNA molecules but also short hairpin RNAs (shRNAs), i.e. fold-back stem-loop structures that give rise to siRNA after intracellular processing, can induce RNA interference [118,119]. This opened up the possibility of constructing vectors expressing the interfering RNA for long-term silencing of gene expression in mammalian cells (summarized in [117,120]). Short hairpin RNA was transcribed using RNA polymerase III promotors that normally control the transcription of either the small nuclear RNA U6 [118,119,121,122] or the H1 RNA component of RNase P [123]. Alternatively, two short RNA molecules were transcribed separately using two U6 promotors [118,124,125]. Vector-mediated expression of siRNA allows the analysis of loss-of-function phenotypes that develop over a longer period of time. In stably transfected cells, silencing was observed even after two months [123].

An alternative approach to prolong siRNA-mediated inhibition of gene expression is the introduction of modified nucleotides into chemically synthesized RNA, despite the fact that even unmodified short double-stranded RNA revealed an unexpectedly high stability in cell culture and *in vivo*. For certain applications, however, further enhancement of the siRNA stability might be desirable. Therefore, modified nucleotides were introduced to the ends of both strands [126]. A siRNA with two 2′-*O*-methyl RNA nucleotides at the 5′ end and four methylated monomers at the 3′ end was as active as its unmodified counterpart and led to a prolonged silencing effect in cell culture. Extension of the methylated stretch of nucleotides as well as the introduction of nucleotides with a bulky 2′-allyl substituent resulted in decreased siRNA activity.

For the first *in vivo* studies of RNA interference in mammals the siRNA or a plasmid coding for shRNA was delivered using rapid injection of a large volume of physiological solution into the mouse tail vein [127,128]. Expression of reporter genes that were either encoded on cotransfected plasmids or in transgenic mouse strains could efficiently be inhibited in most of the organs. In addition, the *Fas* gene has been targeted as an endogenous, therapeutically relevant target for liver injury [129]. After siRNA injection, the Fas mRNA and protein levels were reduced in mouse hepatocytes for 10 days. Silencing Fas protected mice from fulminant hepatitis induced by injection of agonistic Fas-specific antibody; 82% of mice treated with siRNA survived the 10 days of observation, whereas all control animals died within three days.

The high-pressure delivery technique used in the studies described above is, however, a rather harsh method that might influence results and cannot be used for therapeutic applications. Therefore, methods known from standard gene therapy have been adapted for RNA interference. A retroviral vector was used to deliver siRNA that inhibited the carcinogenic *K-ras* allele in human pancreatic tumor cells [130]. Down-regulation of *K-ras* expression in carcinoma cells abolished their ability to form tumors after subcutaneous injection into athymic nude mice. This study also demonstrated the high specificity of siRNA, as only the carcinogenic *K-ras* but not the wild type *K-ras* allele, which differs by only one base pair, was silenced. Furthermore, GFP expression could be suppressed in the brain of transgenic mice after injection of adenovirus vectors expressing siRNA into the striatal region [131]. Activity of endogenous β-glucoronidase could be decreased by injecting recombinant adenoviruses into the mouse tail vein. Interestingly, an RNA polymerase II expression cassette with a CMV promoter and a minimal poly(A) was used for the latter experiments, opening the door to design tissue-specific or inducible siRNA vectors.

Taken together, first promising *in vivo* experiments with siRNA have already been performed and further therapeutically important genes are expected to be targeted soon. No toxic reactions after siRNA application have been observed in the studies performed to date, but great care has to be taken to rule out severe side-effects of long-term induction of RNAi before trials can be started to treat human diseases. Because silencing of gene expression by siRNAs is similar to traditional antisense technology, researchers will be able to benefit from the lessons learned for more than a decade such as the requirement to use proper controls to proof a specific knock-down of gene expression and a careful analysis of possible unspecific effects mediated by the immune system.

Summary

After a long period of ups and downs, antisense technologies have gained increasing attention in recent years. Major improvements have been achieved by the development of modified nucleotides that provide high target affinity, enhanced biostability and low toxicity. As most of the new DNA analogs do not induce RNase H cleavage, the design of antisense-oligonucleotides has to be adjusted depending on whether the target mRNA has to remain intact, e.g. for alteration of splicing, or should be degraded (gapmer technology). Stable ribozymes with high catalytic activity were obtained by systematically modifying naturally occurring ribozymes or by *in vitro* selection techniques. Several antisense-oligonucleotides and ribozymes are currently being investigated in clinical trials and one antisense drug was approved in 1998. A major breakthrough was the discovery that short double-stranded RNA molecules can be used to silence gene expression specifically in mammalian

cells. This method has a significantly higher efficiency compared to traditional antisense approaches and some promising *in vivo* data have already been presented. Therefore, antisense technologies can be expected to be widely used for studies of genes with unknown function, for target validation in drug development and finally, of course, for therapeutic purpose.

Acknowledgements

The author wishes to thank Volker A. Erdmann for his support and advice and Arnold Grünweller, Steffen Schubert, Erik Wade and Harry Kurreck for critical reading of the manuscript. I especially thank all members of my lab for their research in the antisense field. Financial support of the author's work by the Fonds der Chemischen Industrie and by grants to Volker A. Erdmann from the Bundesministerium für Bildung und Forschung (grant 01GG9818/0) and the National Foundation for Cancer Research (NFCR, USA) is gratefully acknowledged. In addition, I want to thank Proligo (Boulder, CO, USA) for supplying locked nucleic acids.

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