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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.

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

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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.

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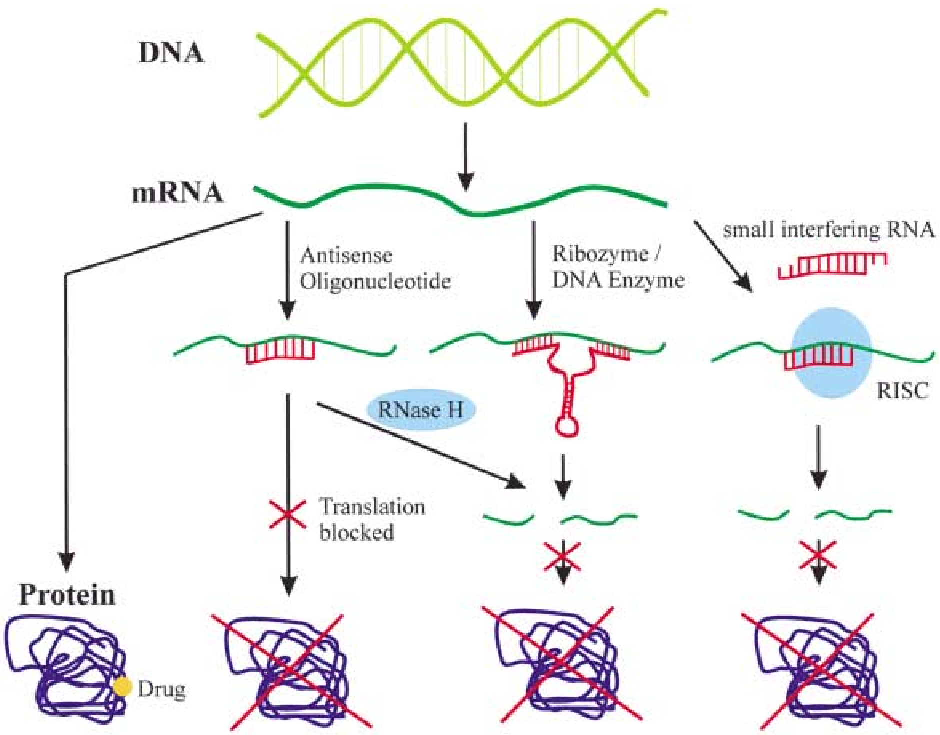

Fig. 1. Comparison of different antisense strategies. While most of the conventional drugs bind to proteins, antisense molecules pair with their complementary target RNA. Antisense-oligonucleotides block translation of the mRNA or induce its degradation by RNase H, while ribozymes and DNA enzymes possess 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

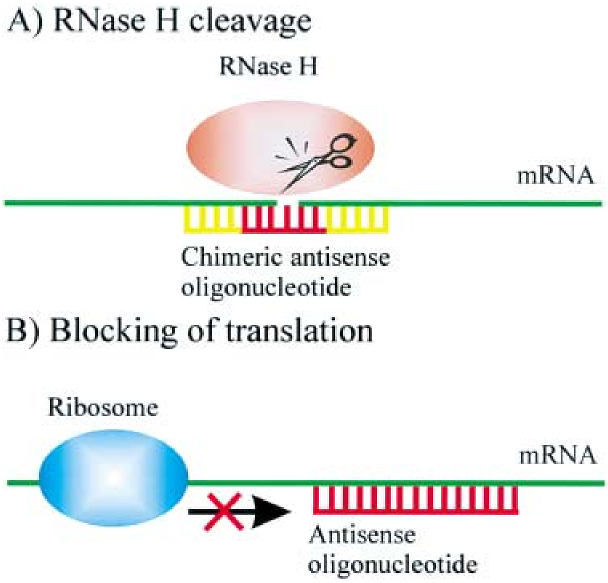

Fig. 2. Mechanisms of antisense activity. (A) RNase H cleavage induced by (chimeric) antisense-oligonucleotides. (B) Translational arrest by blocking the ribosome. See the text for details.

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

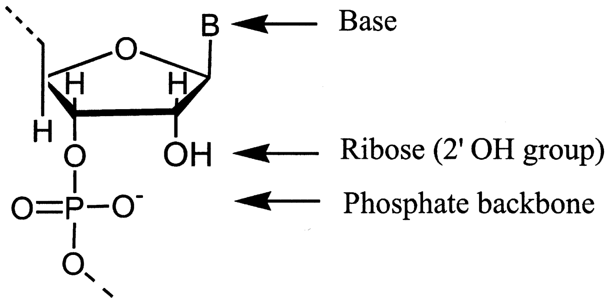

Fig. 3. Sites for chemical modifications of ribonucleotides. B denotes one of the bases adenine, guanine, cytosine or thymine.

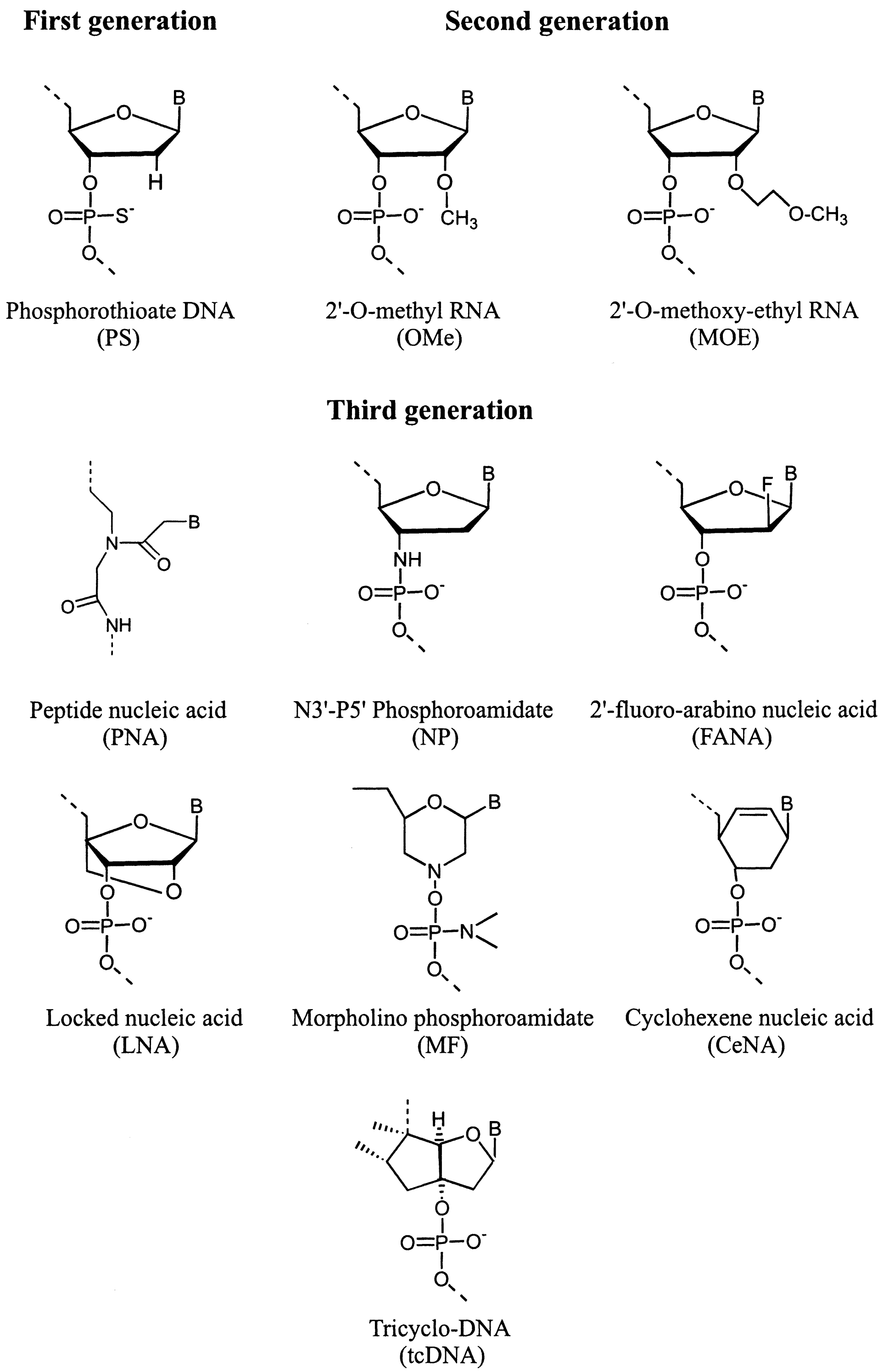

Fig. 4. Nucleic acid analogs discussed in this review. B denotes one of the bases adenine, guanine, cytosine or thymine.

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

PS DNAs have a half-life in human serum of approximately 9–10 h compared to ≈ 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

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 codownregulated 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.

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