CMLS, Cell. Mol. Life Sci. 57 (2000) 1440–1456
1420-682X/00/101440-17 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Review

Synthetic oligonucleotides as RNA mimetics: 2′-modified RNAs and N3′ → P5′ phosphoramidates

M. Egli[a] and S. M. Gryaznov[b,*]

[a]Department of Molecular Pharmacology and Biological Chemistry and The Drug Discovery Program, Northwestern University Medical School, Chicago (Illinois 60611, USA)
[b]Geron Corporation, Nucleic Acids Chemistry, 230 Constitution Drive, Menlo Park (California 94025, USA), Fax + 1 650 473 7750, e-mail: sgryaznov@geron.com

Received 27 March 2000; received after revision 3 May 2000; accepted 5 May 2000

Abstract. Significant interest in synthetic DNA and RNA oligonucleotides and their analogues has marked the past two decades of research in chemistry and biochemistry. This attention was largely determined by the great potential of these compounds for various therapeutic applications such as antisense, antigene and ribozyme-based agents. Modified oligonucleotides have also become powerful molecular biological and biochemical research tools that allow fast and efficient regulation of gene expression and gene functions in vitro and in vivo. These applications in turn are based on the ability of the oligonucleotides to form highly sequence-specific complexes with nucleic acid targets of interest. This review summarizes recent advances in the design, synthesis, biochemical and structural properties of various RNA analogues. These comprise 3′-modified oligonucleotide N3′ → P5′ phosphoramidates, analogues with modifications at the 2′-position of nucleoside sugar rings, or combinations of the two. Among the properties of the RNA minetics reviewed here are the thermal stability of their duplexes and triplexes, hydrolytic resistance to cellular nucleases and biological activity in in vitro and in vivo systems. In addition, key structural aspects of the complexes formed by the RNA analogues, including interaction with water molecules and ions, are analyzed and presented.

Key words. RNA mimetic; oligonucleotide N3′ → P5′ phosphoramidate; sugar-base modification; structure; thermal stability.

Introduction

The versatility and biological importance of RNA molecules have recently transformed these compounds into an important subject of numerous research endeavors. The ability of RNA molecules to fold into well-defined secondary structures imparts to some RNA compounds enzyme-like qualities, including natural phosphodiester backbone cleavage and ligation capabilities [1–4]. The RNA-cleaving properties of exogenous and hydrolytically stable synthetic or endogenously expressed RNA ribozymes have presented a great opportunity for the development of potentially widely applicable RNA-based therapeutic agents. Several clinical trials with ribozyme-based therapeutic agents have been recently initiated. Moreover, RNA molecules have been demonstrated to be capable of performing additional catalytic functions, such as peptide bond formation and DNA cleavage [5–7]. Natural RNA oligomers can also be selected based on their folding into spatially diverse structures which specifically and efficiently recognize various proteins or nucleotide derivatives [8–10].

* Corresponding author.

A significant amount of work has been directed at preparing a multitude of hydrolytically stable RNA analogues for a variety of purposes, ranging from biological probes to potential therapeutic antisense agents and multiple diagnostic applications. Many phosphodiester and phosphorothioate RNA derivatives with a host of 2′-modifications have been synthesized with the goal of increasing the hydrolytic stability and hybridization properties of the RNAs [11, 12]. Additionally, several pyranosyl-RNA analogues have been successfully prepared by Eschenmoser's group [13, 14] to investigate the 'chemical etiology' of nucleic acid structures. Recently, very interesting RNA analogues with conformationally locked sugar rings (LNAs) were described. These compounds form exceptionally stable complexes with RNA complements and are resistant to nucleases [15, 16].

In addition to the above 2′-modifications, an alternative strategy for generating RNA mimetics has been employed. In this approach, conversion of 2′-deoxynucleoside-containing oligonucleotides into structural and functional analogues of natural RNA molecules is achieved via substitution of bridging 3′-oxygen atoms by 3′-amino groups. The properties of the resulting oligodeoxyribonucleotides with internucleoside N3′→P5′ phosphoramidate linkages were recently described [17]. These biopolymers adopt RNA-like C3′-endo or N-type nucleoside sugar puckering, and in general are structurally and functionally similar to the natural RNA molecules.

In this review we will focus on the main physical-chemical, structural, and some biological properties of these RNA analogues and their potential applications.

2′-Modified RNA analogues

An increasing interest in synthetic oligonucleotides and their analogues has marked the past two decades. This attention was largely determined by the potential therapeutic applications of these compounds based on their ability to form specific complexes—primarily duplexes and triplexes—with nucleic acid targets of interest. The high specificity and selectivity of these interactions are governed mainly by the formation of proper and well-defined Watson-Crick and Hoogsteen hydrogen bonds between the heterocyclic bases of targeted nucleic acids and the oligonucleotide ligands [18]. These types of selective recognition and the resultant functional inhibition of the genetic information carriers can potentially open promising new opportunities in nucleotide sequence-based rational drug design and drug discovery. Modified oligonucleotides may also become powerful molecular biological and biochemical research tools which can allow fast and efficient regulation of gene expression and study of gene functions in vitro and in vivo.

However, several important issues remain to be resolved before oligonucleotides may become widely used unique and specific pharmaceutical agents. Among these are: increased thermodynamic stability of the complexes formed by the oligomers with their nucleic acid targets, specificity of the interactions, enhancement of oligonucleotide resistance to enzymatic degradation and hydrolytic stability and bioavailability in cells and in animal model systems and, importantly, favorable pharmacokinetics and biodistribution in human tissues and organs. Additionally, chemical structures of the therapeutic oligonucleotides and the proper choice of suitable and biologically important molecular targets, as well as delivery methods for administration of the compounds, may play a crucial role in ensuring the success of oligonucleotide-based therapeutic approaches.

For quite some time natural RNA oligonucleotides, in general, were known to form more stable complexes with complementary RNA strands than do isosequential DNA molecules [ref. 19 and references therein]. The higher thermal stability of the complexes was attributed to the N-type sugar conformations of both strands, as well as to favorable hydration patterns of RNA duplexes [20, 21]. Consequently, RNA-derived oligonucleotides may be preferable for hybridization-based applications. However, the hydrolytic instability of RNA prompted chemists to search for more resistant RNA analogues potentially suitable for therapeutic applications. To achieve higher stability against nuclease degradation, numerous 2′-modified RNA oligonucleotides, containing a variety of 2′-O-alkyl substituents have been designed, prepared, and evaluated (fig. 1). Physical-chemical and biological properties of these compounds have been thoroughly reviewed in the literature, notably in a recent comprehensive article [22]. In summary, the thermal stabilities of DNA/RNA as well as RNA/RNA complexes formed by the RNA analogues with 2′-O-alkyl groups are usually higher or comparable to those of the natural RNA or DNA compounds. Among the simple 2′-O-alkyl substituted oligomers, 2′-O-methyl compounds form the most thermodynamically stable complexes with RNA, with values for ΔT_m of approximately 1.5 °C and 0.5 °C per modification relative to isosequential natural DNA and RNA strands, respectively [23]. The thermal stability of the complexes is reduced as the length of the 2′-O-alkyl chain increases, and 2′-O-pentyl derivatives are as stable as their 2′-deoxy counterparts. Among the 2′-O-alkyl derivatives, some of the most stable complexes are formed by 2′-O-(2-methoxyethyl) (2′-O-MOE)-containing RNA analogues, with values for ΔT_m of $\sim$1.6 °C per modified nucleotide. Resistance to enzymatic hydrolysis for 2′-O-alkyl RNA derivatives is concomi-

tantly increased with the length of the 2′-O-substituents. Thus, 2′-O-MOE-modified oligonucleotides and, in particular, zwitterionic (at physiological pH) oligonucleotides with 2′-O-(3-aminopropyl) (2′-O-AP) modifications are practically completely resistant to cellular nuclease digestion [24–26]. 2′-O-alkyl-modified oligonucleotides retain native RNA-like C3′-endo or N-type sugar puckering, and form A-type double helixes with complementary RNA strands. Structural aspects of these molecules and their complexes will be discussed later in the review.

Hybrid duplexes between RNA and 2′-O-alkyl RNA compounds, the latter containing either natural phosphodiester or modified phosphorothioate backbones, are not substrates for RNase H. The generally lower biological activity of 2′-O-alkyl RNAs relative to their DNA phosphorothioate counterparts in antisense applications has in part been attributed to this shortcoming [27]. However, chimeric molecules, or so-called 'gapmers,' containing five or more 2′-deoxy phosphodiester or phosphorothioate nucleotides in a central region flanked by 2′-O-alkyl derivatives, have demonstrated high sequence-specific antisense activity in various in vitro and in vivo model systems [28, 29]. These oligonucleotides, unlike the uniformly 2′-O-modified compounds, form complexes that are substrates for RNase H-mediated RNA hydrolysis. In addition, several hydrolytically active ribozyme-like molecules have been prepared which feature a natural phosphodiester RNA catalytic core and RNA recognition elements with 2′-O-alkyl- or 2′-O-allyl-protected nucleosides [30, 31]. These compounds demonstrated high RNA-cleaving catalytic activity, while being resistant to digestion by cellular nucleases, and are currently under evaluation in numerous clinical trials [32]. Moreover, uniformly modified 2′-O-methyl RNA oligomers demonstrated utility for applications that do not require cleavage of the RNA targets, such as steric blocking of undesirable RNA splice sites [33], or for construction of dumbbell-like DNA/RNA chimeric duplexes which are being used successfully in promising DNA-editing experiments [34, 35].

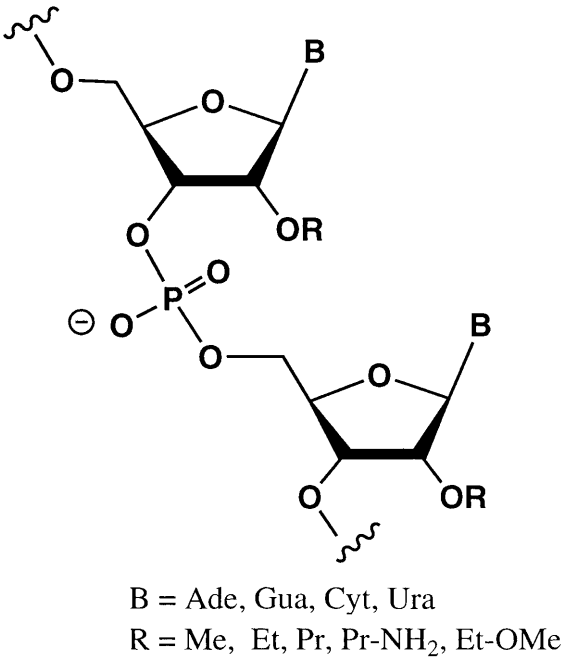

Figure 1. Structures of selected 2′-O-modified RNAs (Pr-NH_2, 3-aminopropyl; Et-Ome, 2-methoxyethyl).

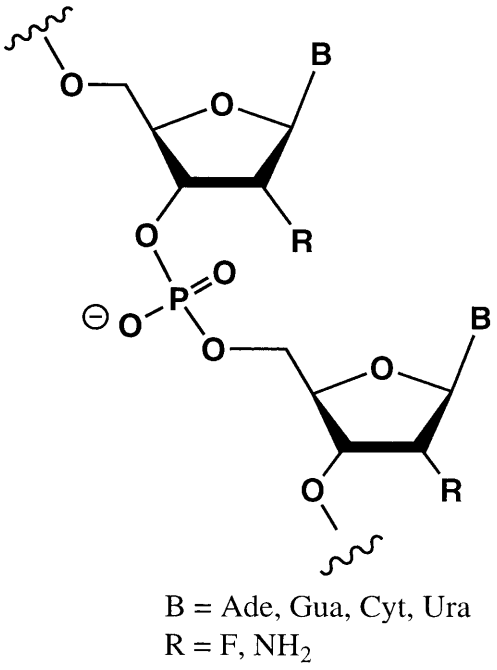

Figure 2. Structures of 2′-deoxy-2′-fluororibonucleic acid (R = F) and 2′-deoxy-2′-aminoribonucleic acid (R = NH_2).

Several other interesting classes of RNA mimetics that contain 2′-substituents other than 2′-O-alkyl or 2′-O-alkoxyalkyl groups have been prepared and studied (fig. 2). Among these analogues, 2′-fluoro-substituted RNA probably constitutes one of the most intriguing classes of compounds. These oligonucleotides can also be considered as DNA analogues, whereby the 2′-hydrogen is replaced by a fluorine atom. The 2′-fluoro-substituted compounds are among the most thermodynamically stable complexes with complementary nucleic acids among all 2′-modified RNAs. The increase in the melting temperature of duplexes reaches up to 2 °C or

2.3 °C per substitution for phosphodiester or phosphorothioate oligonucleotides, respectively [36]. At the same time, these oligonucleotides do not resist enzymatic degradation by cellular nucleases, and their stability is similar to that of native DNA [36, 37]. The sugar pucker of the 2′-fluoro nucleosides is of the C3′-endo type. Uniformly modified oligonucleotides containing 2′-fluoro nucleosides form A-type duplexes with their RNA complements that are structurally very similar to the parent RNA/RNA complexes [36]. Interestingly, 2′-fluoro polyadenylic acid can be efficiently used as mRNA, similar to poly-r(A), for the synthesis of poly-(Lys) in vitro [38]. This finding strongly suggests that the presence of 2′-hydroxyl groups is not crucial for ribosomal recognition of mRNA molecules. The structural similarities with native RNA that are conferred by the presence of 2′-fluoro nucleosides probably allow recognition of 2′-fluoro RNA by the ribosomal machinery and its translation into poly-(Lys) protein.

In other applications, 2′-fluoro as well as 2′-aminopyrimidine nucleosides have been introduced at various positions of synthetic ribozymes [39, 40]. In general, these modifications did not significantly reduce ribozyme catalytic activity, despite the preference of 2′-amino nucleosides for the S-type sugar conformation. At the same time, resistance to nuclease degradation of these 2′-fluoro- and 2′-amino-containing compounds was increased by at least a 1000-fold relative to unmodified ribozymes. Moreover, hydrolytically stable aptamers that contain 2′-fluoro- and 2′-amino-modified nucleosides and display high affinities for proteins were isolated using the SELEX technique [41].

Oligonucleotide N3′ → P5′ phosphoramidates: duplex and triplex formation properties and biological activity

Uniformly modified oligo-2′-deoxyribonucleotides containing internucleoside N3′ → P5′ phosphoramidate linkages have been synthesized and described (fig. 3) [17, 42]. These compounds contain 3′-amino-2′-deoxynucleosides, and the nucleosides are linked together in oligonucleotides via phosphoramidate monoester linkages. Thus, they differ structurally in an important way from native DNA and RNA compounds, in which nucleosides are connected through phosphodiester groups. Similar to the phosphodiester oligomers, oligonucleotide N3′ → P5′ phosphoramidates are ionized and negatively charged at neutral pH, and their internucleoside phosphorus atoms are all achiral. 2′-Substituted oligonucleotide N3′ → P5′ phosphoramidates containing 2′-fluoro-3′-amino- or 2′-hydroxyl-3′-aminonucleosides connected via phosphoramidate linkages have also been recently described [43–45]. These oligomers may also be considered structural analogues of native DNA and RNA molecules (fig. 3).

Oligo-2′-deoxynucleotide N3′ → P5′ phosphoramidates form very stable duplexes with complementary DNA and RNA strands. Increases in the duplex melting temperature reached up to 0.7–1.2 °C and 2.3–2.6 °C per modified nucleoside unit for the complexes formed with DNA and RNA, respectively, relative to the isosequential phosphodiester oligonucleotides [17, 42]. Duplex formation by the oligonucleotide phosphoramidates was shown to be a highly sequence-specific process that is guided by proper Watson-Crick hydrogen bond formation. Incorporation of a single mismatched nucleoside in the complementary strand usually reduces the melting temperature of the complexes by more than 12 °C for a model 11-mer [17].

Compared to their phosphodiester counterparts, these compounds also exhibit a different chromatographic behavior under reverse-phase (RP), ion exchange (IE), and gel electrophoresis conditions. Phosphoramidate mobility is noticeably higher on RP columns, and much lower on IE columns. Also, under slab and capillary gel electrophoresis conditions, oligonucleotide phosphoramidates demonstrate lower mobility than their phosphodiester DNA counterparts [46, 47]. All these differences indicate the significantly increased hydration and rigidity of the N3′ → P5′ phosphoramidate sugar-phosphate backbone relative to native phosphodiester

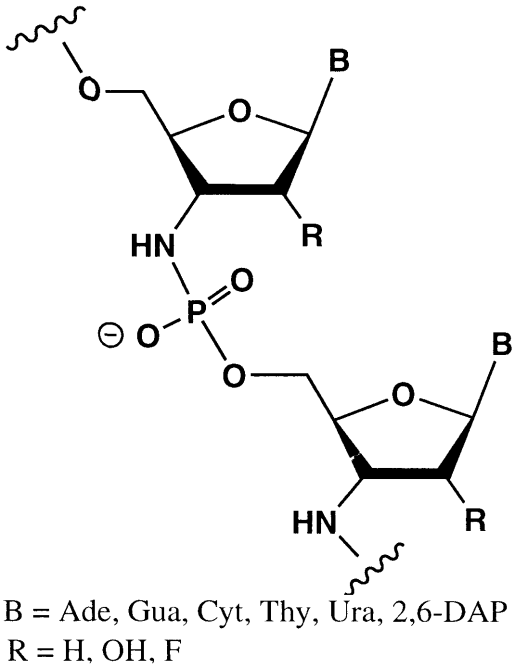

Figure 3. Structures of N3′ → P5′ phosphoramidate DNA (R = H) and RNA (R = OH) and N3′ → P5′ phosphoramidate 2′-deoxy-2′-fluororibonucleic acid (R = F) (2,6-DAP, 2,6-diaminopurine).

oligomers. These results were supported by the X-ray crystallographic structure analysis of a phosphoramidate DNA duplex [ref. 48, and see text below].

High-resolution proton nuclear magnetic resonance (NMR) experiments demonstrated that the 3′-aminonucleoside 2′-deoxyfuranose conformation is predominantly of the C3′-endo or N-type in free 3′-aminonucleosides and in 3′-aminonucleosides incorporated into single- and double-stranded oligonucleotide N3′→P5′ phosphoramidates [49, 50]. Moreover, circular dichroism (CD) spectra of phosphoramidate DNA duplexes were consistent with formation of an RNA-like A-form conformation [17]. This change in the helix geometry compared with DNA is likely due to N-type sugar puckering of 3′-aminonucleosides.

These findings were confirmed by the X-ray analysis of crystals of the self-complementary Dickerson-Drew dodecamer CGCGAATTCGCG containing only N3′→P5′ internucleoside phosphoramidate linkages [48]. This phosphoramidate duplex adopts an RNA-like A-type double helix, despite being made from 2′-deoxynucleosides. The X-ray analysis also revealed a more extensive hydration pattern of the oligonucleotide phosphoramidate sugar-phosphate backbone compared to that for phosphodiesters. In addition, the 3′-amino groups mediate water bridges between opposite strands across the minor groove. These results will be discussed in further detail below.

Non-self-complementary oligo-2′-deoxynucleotide N3′→P5′ phosphoramidates also form extremely stable duplexes with each other. The melting temperatures of these complexes are usually up to 30 °C higher for 12- to 16-mers than those for the isosequential phosphodiester DNA and RNA compounds [42, 46].

2′-Modified oligonucleotide N3′→P5′ phosphoramidates with 2′-fluoro or 2′-hydroxyl groups in a ribo-configuration form even more thermally stable duplexes with DNA and RNA strands than their parent 2′-deoxy compounds. Thus, the melting temperatures of the duplexes formed by the 2′-fluoro and 2′-hydroxyl decathymidylic N3′→P5′ phosphoramidates with poly(A) were approximately 17 °C and 6 °C higher, respectively, than that of their 2′-deoxy phosphoramidate counterpart [43–45]. The 2′-fluoro- and 2′-hydroxyl substituted 3′-aminonucleosides apparently have higher populations of N-type nucleoside conformations. This conclusion is based on analysis of the nucleoside sugar ring H1′-H2′ coupling constants, which were derived from the proton NMR spectra of mono- and di-nucleotides. This difference likely contributes to the higher thermal stability of the 2′-modified phosphoramidate duplexes. Presumably, better hydration of the sugar-phosphate backbone of these oligomers also contributes to the increased stability. These 2′-fluoro and 2′-hydroxyl substituted oligo-N3′→P5′ phosphoramidates are considerably more acid resistant than their 2′-deoxy counterparts. This is likely due to the electron-withdrawing effects of 2′-fluoro and 2′-hydroxyl groups that reduce the basicity of the 3′-nitrogen atom.

Oligopyrimidine-containing oligonucleotide N3′→P5′ phosphoramidates form very stable triplexes with oligopurine:oligopyrimidine segments of phosphodiester duplexes. This was demonstrated by various experimental techniques, including thermal dissociation, gel-shift analysis, cross-linking experiments, endonuclease restriction arrest, and fourier transform infrared (FTIR) spectroscopy [17, 43, 51–53]. Thus, the melting temperature of a triplex between phosphoramidate decathymidylate and a phosphodiester duplex was 47.2 °C under near physiological pH and salt conditions [17]. An even higher triplex stability was observed for the d(T,C)-containing phosphoramidate CTTCTTCCTTA, where the triplex T_m value was 62 °C. Parent phosphodiester oligonucleotides failed to form triplexes under similar experimental conditions [42]. Another d(T,C)-containing phosphoramidate oligonucleotide, TTTCCTCCTCT, formed a much more stable triplex with a double-stranded DNA target than did isosequential phosphodiester-linked DNA, RNA, or 2′-O-methylated oligomers. The triplex T_m values were 45, < 10, 26, and 26 °C, respectively. Similar to the effect on phosphodiester oligonucleotides, C5-methylation of cytosine bases of the phosphoramidates increased their triplex stability, and the T_m value for the triplex formed by oligonucleotide $TTTC^{Me}C^{Me}TC^{Me}C^{Me}TC^{Me}T$ was 61 °C [51, 52].

Among the natural phosphodiester nucleic acids, pyrimidine-containing RNA oligomers form the most stable triplexes with DNA duplexes. In these triplexes, the third or Hoogsteen strand assumes a parallel orientation relative to the targeted purine strand of the duplex [54]. Their higher stability was attributed, at least in part, to the N-type sugar puckering of ribonucleosides, which may facilitate and stabilize Hoogsteen-type base pairing. It is important to note that oligonucleotide phosphoramidates that also feature RNA-like N-type sugar conformation of nucleosides form even more stable triplexes than the corresponding RNA phosphodiesters. Moreover, 2′-O-methyl-modified analogues with a phosphodiester backbone that also adopt an N-type sugar conformation form less stable triplexes than the isosequential and conformationally similar 2′-deoxy or 2′-ribo-N3′→P5′ phosphoramidates. Interestingly, 2′-fluoro d(C,U)-containing phosphodiester oligonucleotides were reported to form the least stable triplexes relative to the 2′-deoxy, 2′-O-methyl or 2′-ribo counterparts. The melting temperatures for their triplexes at pH 6.1 were 20.7, 28.3, 36.8, and 37.7 °C, respectively [55]. The stability of triplexes comprising 2′-fluoro phosphodiester oligonucleotides is reduced despite the prefer-

ence for an N-type sugar conformation of 2′-fluoronucleosides. This indicates that some additional factors besides the nucleoside sugar conformation may contribute to the elevated or diminished stability of individual triplexes. These factors could include, for example, sugar phosphate backbone hydration and rigidity, spatial positioning of the nucleoside bases, and changes in the basicity of the heterocycles caused by the sugar ring substitutions and modifications. Oligopyrimidine N3′→P5′ phosphoramidates may have an optimal combination of triplex-favoring properties, such as N-type sugar puckering and increased hydration of the sugar phosphate backbone due to the presence of 3′-aminogroups. Similar to the natural purine-rich oligoribonucleotides, d(A,G)-containing N3′→P5′ phosphoramidates seem unable to form stable triplexes with antiparallel orientation to the polypurine strand of DNA duplexes [51]. The C3′-endo sugar puckering of nucleosides that is preferred by oligonucleotide N3′→P5′ phosphoramidates and by native RNA probably disfavors formation of the required reverse Hoogsteen-type base pairs between the targeted DNA oligopurine strand and the triplex-forming compounds. In contrast, oligonucleotide N3′→P5′ phosphoramidates containing blocks of thymidine and guanosine nucleotides form even more stable triplexes than do d(T,C)-containing compounds with the same double-stranded DNA target when the oligonucleotides were oriented in a parallel fashion to the oligopurine strand of the duplex [51]. Interestingly, pentadecathymidylic phosphoramidate formed only a 2T:1A triplex with 2′-deoxy pentadecaadenylate, even if the molar ratio between the purine and pyrimidine strands was kept at 1:1, as demonstrated by gel shift analysis, thermal dissociation experiments, and FTIR spectroscopy [53].

Oligonucleotide N3′→P5′ phosphoramidates are highly active in various biological systems. They exhibit high RNA target selectivity and sequence-specific activity as antisense and antigene agents under various in vitro and in vivo conditions [56–59]. The reported results demonstrate their potential application as very efficient and sequence-specific agents in vitro and in vivo, with an observed activity superior to that for the other reference compounds. The differences in cellular uptake, bioavailability, and much higher thermodynamic stability of the formed complexes may be the key factors contributing to the better biological activity of oligonucleotide phosphoramidates.

Oligonucleotide N3′→P5′ phosphoramidates as functional mimetics of RNA molecules

As described before, oligonucleotide N3′→P5′ phosphoramidates in single- and in double-stranded arrangements are structurally similar to their natural phosphodiester RNA counterparts. Consequently, functionally important natural RNA structural elements may plausibly be prepared from oligonucleotide phosphoramidates and used as synthetic decoys for RNA-binding proteins.

This idea was tested in a model system entailing RRE and TAR RNA structural elements of HIV-1. These elements bind to the HIV-1 Rev and Tat proteins, respectively, and their interaction plays an important role in regulating viral proliferation. Thus, several oligonucleotide phosphoramidates isosequential to the RNA strands composing the RRE and TAR elements were prepared. These oligonucleotides can form bulged homoduplexes with each other. Furthermore, these oligomers form heteroduplexes with native phosphodiester RNA strands. The nucleotide composition of these complexes corresponds to the RRE or TAR segments that are recognized by the cognate Rev and Tat proteins [60]. This recognition is crucial for the viral replication process. The duplexes with incorporated N3′→P5′ phosphoramidate building blocks were structurally similar to the native all-RNA elements as determined by CD spectroscopy. More important, the complexes, composed of either two phosphoramidate strands or one phosphoramidate and one phosphodiester RNA strand, were recognized by the corresponding Rev- or Tat-derived peptides. The affinities of the peptides for the modified duplexes were practically the same as those for the native all-RNA substrates, as shown by gel shift analysis of the formed peptide-nucleic acid complexes under non-denaturing conditions [60]. These results are very interesting in view of the lack of 2′-hydroxyl groups in the 2′-deoxy phosphoramidate compounds. The 2′-hydroxyl groups were considered to be an important element for Rev and Tat substrate recognition. The data indicate that for these particular nucleic acid elements, their recognition and binding by the corresponding proteins may not necessarily require the presence of 2′-hydroxyl groups. Instead, a general A-form geometry of the nucleic acid helix and N-type sugar puckering of the composing 3′-amino nucleosides may be more important. The same may be true for some other RNA-protein interactions, where the general shape of the nucleic acid complex plays a crucial role in its recognition. Additionally, binding of Rev- and Tat-derived peptides to phosphoramidate complexes was highly sequence specific.

In contrast to 2′-deoxy phosphoramidate-containing duplexes, complexes formed by the isosequential phosphodiester oligodeoxynucleotides were not recognized by the cognate peptides under the experimental conditions used. These data suggest that oligonucleotide phosphoramidates and their complexes could be used as nucleic acid decoys for RNA-binding proteins and per-

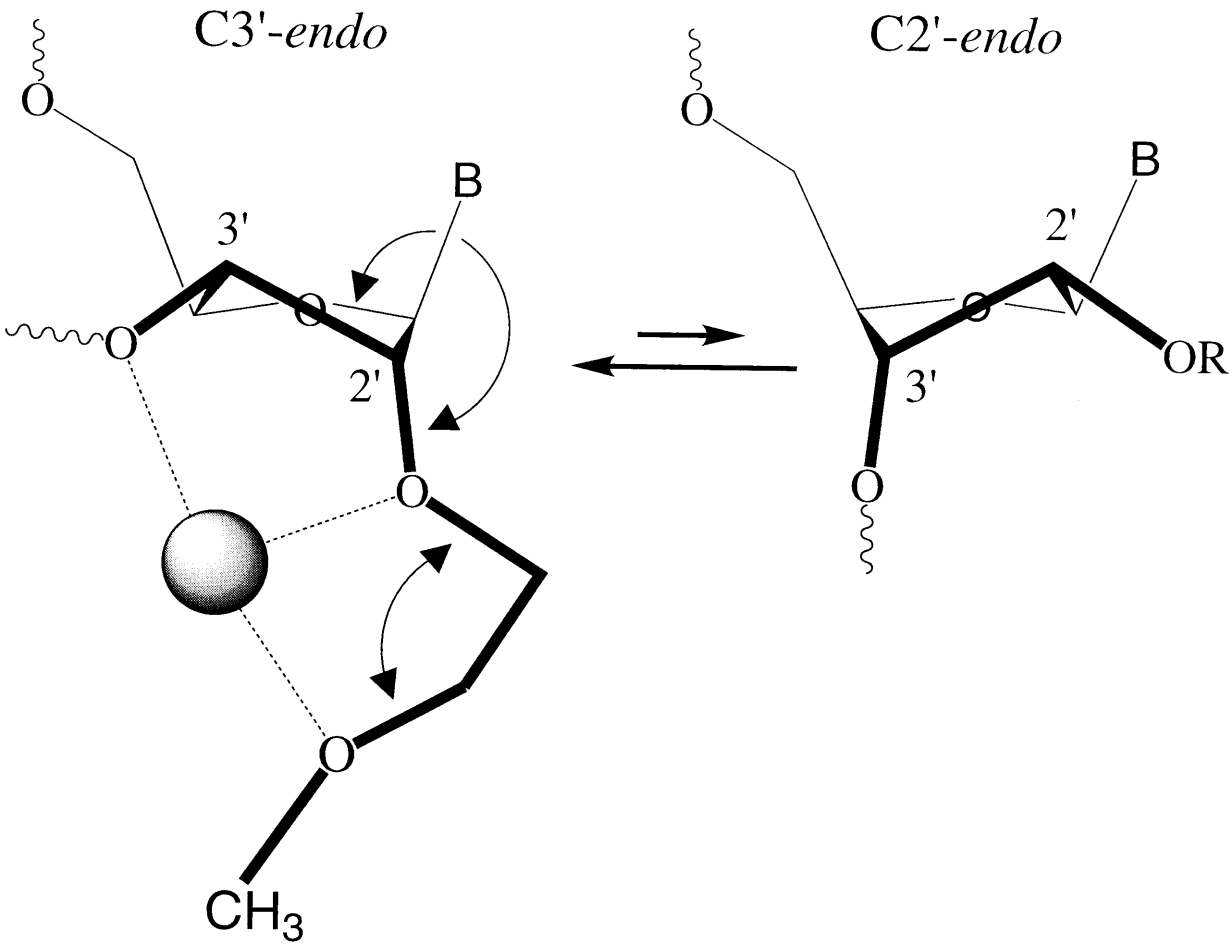

Figure 4. Influence of electronegative 2′- or 2′-O-substituents on the furanose sugar conformational equilibrium. Gauche effects (indicated by curved arrows) and structured water preorganize the sugar for an A-type or C3′-endo conformation [left, using the 2′-O-(2-methoxyethyl)-ribonucleoside as an example]. In oligonucleotides, a B-type or C2′-endo conformation (right) with ribose as well as 2′- and 2′-O-modified analogues is strongly disfavored as a consequence of stereoelectronic and steric effects. A water molecule trapped between the 3′-, 2′- and MOE-oxygen atoms is drawn as a gray sphere and hydrogen bonds are dashed lines.

haps for other classes of compound interacting with RNA. The ability of 2′-deoxyoligonucleotide phosphoramidates to act as RNA substrates can likely be further enhanced by incorporation of 2′-hydroxyl- or 2′-fluoro-3′-amino nucleosides which should render phosphoramidate oligonucleotides even more RNA-like.

Structural investigations of RNA mimetics

The conformations of double-stranded DNA and RNA

Two fundamental geometries are observed with duplex DNA, the A- and the B-forms, the latter often being referred to as the physiological conformation of DNA. Conversely, double-stranded RNA is conformationally more rigid and adopts an A-form geometry. Conformational variation in DNA is driven by the flexibility of the 2′-deoxyribose sugar moiety [61]. Various stereoelectronic effects influence the conformational equilibria of the pentofuranose moiety as described by the pseudorotation phase cycle [62]. In addition, steric factors play an important role in the control of sugar conformation, both at the nucleotide and oligonucleotide levels. For example, the presence of the 2′-hydroxyl group in the backbone of RNA shifts the sugar conformational equilibrium toward the C3′-endo state (fig. 4) [see, for example, ref. 63]. The thermodynamic stability of RNA duplexes exceeds that of RNA-DNA hybrid duplexes of identical sequence [19]. This has important consequences for the design of antisense oligonucleotides targeted against particular portions of messenger RNA. Thus, in terms of RNA affinity, RNA mimetics necessarily will outperform DNA mimetics [20, 64–67].

Preorganizing the pentofuranose moiety for a C3′-endo-type pucker

There are three basic strategies for shifting the conformational equilibrium of the furanose moiety toward the C3′-endo state (fig. 4). The first is modification at the 2′-position with electronegative substituents. The second is replacement of the 3′-oxygen in the backbone by either carbon [ref. 68 and references cited therein] or nitrogen [ref. 42 and references cited therein]. The third strategy aims at conformationally restricting the furanose moiety or even locking its conformation in an RNA-like state, as is the case in LNAs [69]. All three will tilt the balance of a variety of competing stereoelectronic and steric effects in favor of a 3′-endo puckering mode. Beyond the effects on conformation, such chemical modifications will affect hydration and, therefore, the stability of the heteroduplex between oligonucleotide analogue and RNA [20, 70]. In addition, modifications can confer chemical stability, nuclease resistance, and favorable pharmacokinetic and pharmacodynamic properties. Below we will discuss the structural properties of a range of RNA mimetics. Most of the structural insights discussed here are based on the results of X-ray crystallographic investigations of such oligonucleotide analogues in the laboratory of one of the authors.

Structural properties of 2′-O-modified RNA analogues

Structural studies of 2′-O-modified RNAs comprise species with alkyl and alkoxyalkyl substituents (figs 1, 4). The crystal structure of an A-form DNA duplex containing isolated 2′-O-methyl adenosines revealed C3′-endo puckering of the modified riboses and antiperiplanar (*ap*) orientation of the C3′-C2′ and O2′-methyl bonds (fig. 4) [71]. The 2′-oxygens appear to be relatively weak hydrogen bond acceptors. Although the 2′-oxygens of both modified residues formed contacts to water molecules, the corresponding distances were relatively long, suggesting that water may not play a significant role in the higher thermodynamic stability of 2′-O-methyl RNA relative to RNA [23]. This initial finding at medium resolution was later confirmed by the crystal structure of the fully 2′-O-methylated RNA duplex with the sequence CGCGCG determined at 1.3 Å resolution [72]. Thus, the minor groove of the modified duplex featured only a relatively small number of water molecules. Therefore, the structural results have not provided a satisfactory explanation for the superior thermodynamic stability of 2′-O-methyl RNA compared with RNA. Factors that may contribute are the higher conformational rigidity of 2′-O-methyl ribose relative to native ribose and van der Waals-type interactions between methyl group and base atoms in the minor groove.

2′-O-alkoxyalkyl RNAs in which the oxygens of substituents are separated by ethyl groups form duplexes with RNA that exhibit superior thermodynamic stability relative to hybrids between 2′-O-alkyl RNAs and RNA [24, 73–75]. In addition to enhanced RNA affinity, 2′-O-alkoxyalkyl modifications provide resistance to nucleases matching or exceeding that of DNA phosphorothioates [66, 76]. Interestingly, longer 2′-O-alkoxyalkyl substituents such as 2′-O-methyl-[tri-(oxyethyl)] (TOE; fig. 5) or branched species do not impair RNA affinity. This contrasts with the effects of growing chain length on RNA affinity and nuclease resistance for 2′-O-alkyl substituents. In the case of the latter analogues, RNA affinity decreases with growing length of the substituent. However, nuclease resistance is directly correlated with the length of the aliphatic substituent, the 2′-O-nonyl modification being much more stable to degradation than the 2′-O-methyl modification [77].

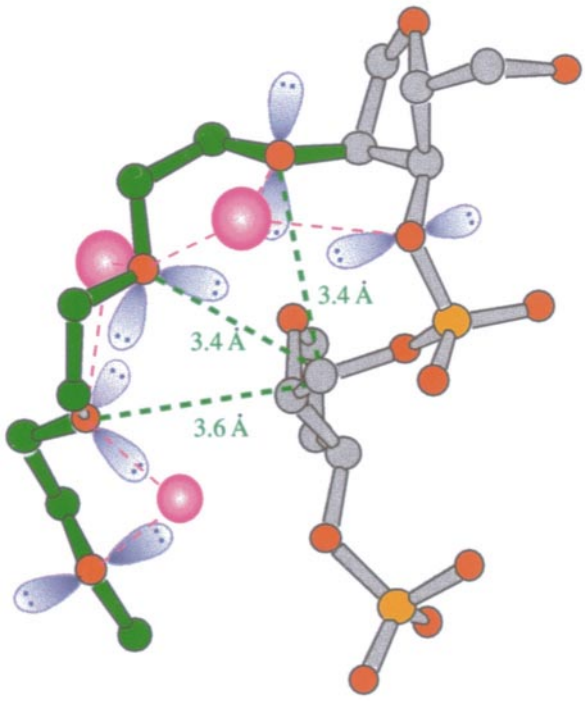

Figure 5. Electrostatic interactions between oxygen atoms from the 2′-O-methyl[tri(oxyethyl)] (TOE) substituent of residue n and the deoxyribose of residue (n + 1) in the crystal structure of a decamer DNA duplex with incorporated 2′-O-TOE thymidines. Atoms are colored gray, green, red, and orange for carbon, TOE carbon, oxygen and phosphorus, respectively. Oxygen lone pairs are shown in blue, anticipated C-H···O hydrogen bonds between the 5′-methylene group and TOE oxygen atoms are dashed lines, with the corresponding C···O distances in Å, and water molecules are drawn as pink spheres.

Two structural studies of 2′-O-alkoxyalkyl-modified nucleic acid fragments have been recently published. In the first, crystal structures of A-form DNA duplexes containing a single 2′-O-alkoxyalkyl-modified thymidine [MOE, TOE, or ethoxymethylene (EOM)] per strand were determined at resolutions between 1.6 and 1.93 Å [78]. While both the 2′-O-MOE and the 2′-O-TOE substituents lead to higher stability of duplexes between modified oligonucleotides and RNA, the 2′-O-EOM substituent, in which O2′ and the outer oxygen atom are separated by a methylene group, has a destabilizing effect [73]. The crystallographic data demonstrated that in the case of the two stabilizing modifications, ribose as well as the 2′-O-alkoxyalkyl substituent are conformationally preorganized for an A-form duplex. The conformations of all torsion angles around ethyl C-C bonds in the substituents are synclinal (*sc*), in accordance with the gauche effect (figs 4, 5). The substituents are well hydrated and a conserved hydration site involved O3′, O2′, and the MOE oxygen (fig. 4). The particular conformations observed with 2′-O-TOE substituents in the crystal structure appear to be stabilized by a network of C-H···O-type hydrogen bonds between substituent and backbone as well as a network of coordinated water molecules (fig. 5). Further conclusions from the structural work are the lack of stabilizing hydration motifs with the thermodynamically unfavorable 2′-EOM substituent and a more promiscuous conformational behavior of this substituent, most likely as a result of the missing gauche effect.

A second structural study focused on the conformational properties of a fully modified 2′-O-MOE RNA duplex [79]. Four structures of the modified duplex with sequence CGCGAAUUCGCG based on crystals grown under a variety of conditions were determined at resolutions up to 1.7 Å. The duplex assumes an overall A-form conformation with a slightly reduced helical rise relative to wild-type A-form RNA (fig. 6). The previous finding that the geometry of the 2′-O-MOE substituent in the minor groove is relatively rigid was largely borne out by these structures. Most substituents feature an *sc* conformation of the torsion around the ethyl C-C bond, and a water molecule forming hydrogen bonds to O2′, O3′, and MOE oxygen is conserved with almost all residues (fig. 7A). This water molecule can stabilize further waters that hydrogen bond to either base atoms or non-bridging phosphate oxygens in the backbone. The presence of the 2′-O-MOE substituent clearly improves the hydration of the sugar-phosphate backbone which may even surpass the excellent hydration of natural RNA due to the ribose 2′-hydroxyl group [21]. In summary, important structural features of 2′-O-MOE RNA such as conformational preorganization of sugar and 2′-O-substituent and an extensive water structure are in line with the increased thermodynamic stability of hybrid duplexes between 2′-O-MOE RNA and RNA relative to the corresponding DNA-RNA hybrids. Moreover, the hydration motif involving MOE substituent and ribose atoms could play a role in the increased nuclease resistance of 2′-O-MOE RNA and the reduced non-specific binding to serum proteins compared with phosphorothioate DNA [76, 79].

Structural properties of N3′ → P5′ phophoramidate DNA

The most detailed picture obtained to date for the structural consequences of replacement of the 3′-oxygen in DNA by an amino group is based on the 2-Å X-ray crystal structure determination of the all-modified N3′ → P5′ phosphoramidate DNA duplex with sequence CGCGAATTCGCG [48]. This particular sequence was selected because it had been used in the first crystal structure determination of a B-form DNA [80]. In principle, any major geometric differences between the structure of this so-called Dickerson-Drew dodecamer and the phosphoramidate DNA dodecamer in our crystal structure can therefore be attributed to the chemical modification in the sugar-phosphate backbone of the latter. Indeed, the phosphoramidate duplex adopts an A-form geometry (fig. 6), consistent with the diminished strength of the gauche effect between the deoxyribose 4′-oxygen and the 3′-nitrogen relative to the corresponding interaction between O4′ and O3′ in DNA. The puckers of all sugars in the modified duplex fall into the C3′-endo conformational range (fig. 4) [70].

As in the case of the 2′-O-alkoxyalkyl-modified duplexes, the presence of the 3′-amino group significantly increases the number of water molecules that are conferred on the sugar-phosphate backbone. The DNA backbone features many hydrogen bond acceptors but is completely deficient of donors. Replacing the 3′-oxygen by an amino group introduces a moiety into the backbone that can act as both a hydrogen bond donor and acceptor. Although the resolution of the structure did not permit us to locate all first-shell water molecules, most of the phosphoramidate moieties can clearly stabilize two to three water molecules (pink spheres in fig. 7B). In many places, two water molecules bridge the amino group and one of the adjacent non-bridging phosphate oxygens. The particular packing arrangement in the rhombohedral lattice of the phosphoramidate DNA dodecamer leads to close lateral contacts between adjacent duplexes. Potentially repulsive electrostatic interactions between tightly spaced phosphoramidate moieties from three duplexes are relieved by ammonium and chloride ions located on the threefold rotation axis present in the space group of the phosporamidate DNA crystals (fig. 8). The chloride anions exhibit a tetrahedral coordination with three

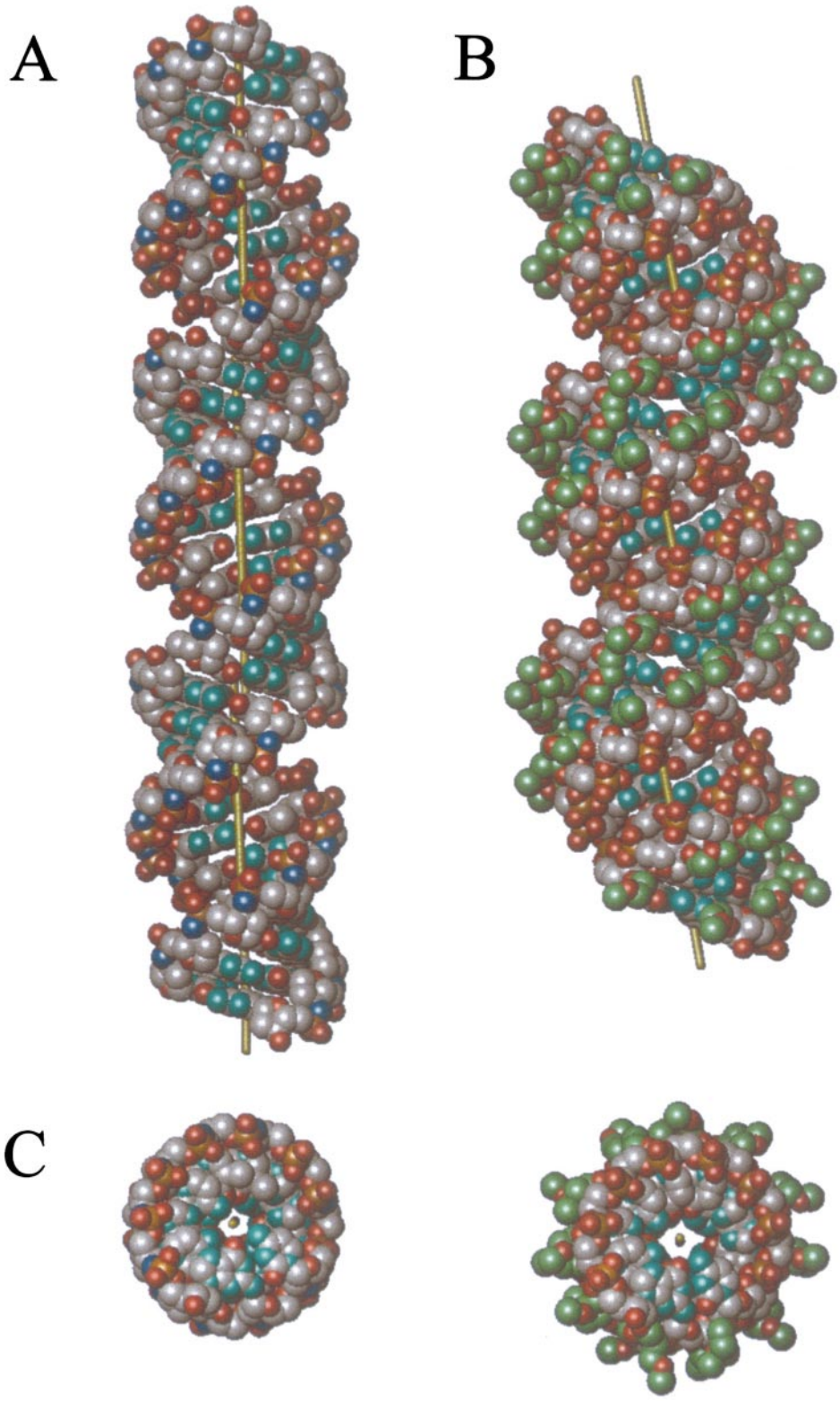

Figure 6. Overall geometries of N3′→P5′ phosphoramidate DNA (*A*, *C*) and 2′-O-(2-methoxyethyl) (MOE) RNA (*B*, *C*) dodecamer duplexes of sequence CGCGAATTCGCG (DNA) and CGCGAAUUCGCG (RNA), viewed perpendicular (*A*, *B*) and roughly along their helical axes (shown in yellow) (*C*). In the phosphoramidate DNA, crystal duplexes are stacked end-to-end, resulting in nearly superimposed local and overall helical axes. In the 2′-O-MOE RNA crystal, individual duplexes are tilted relative to the direction defined by the duplex stack. The projections in (*C*) are along the local helix axis, both for phosphoramidate DNA and 2′-O-MOE RNA. Atoms are colored gray, red, cyan, orange, blue, and green for carbon, oxygen, nitrogen, phosphorus, 3′-nitrogen, and MOE carbon, respectively.

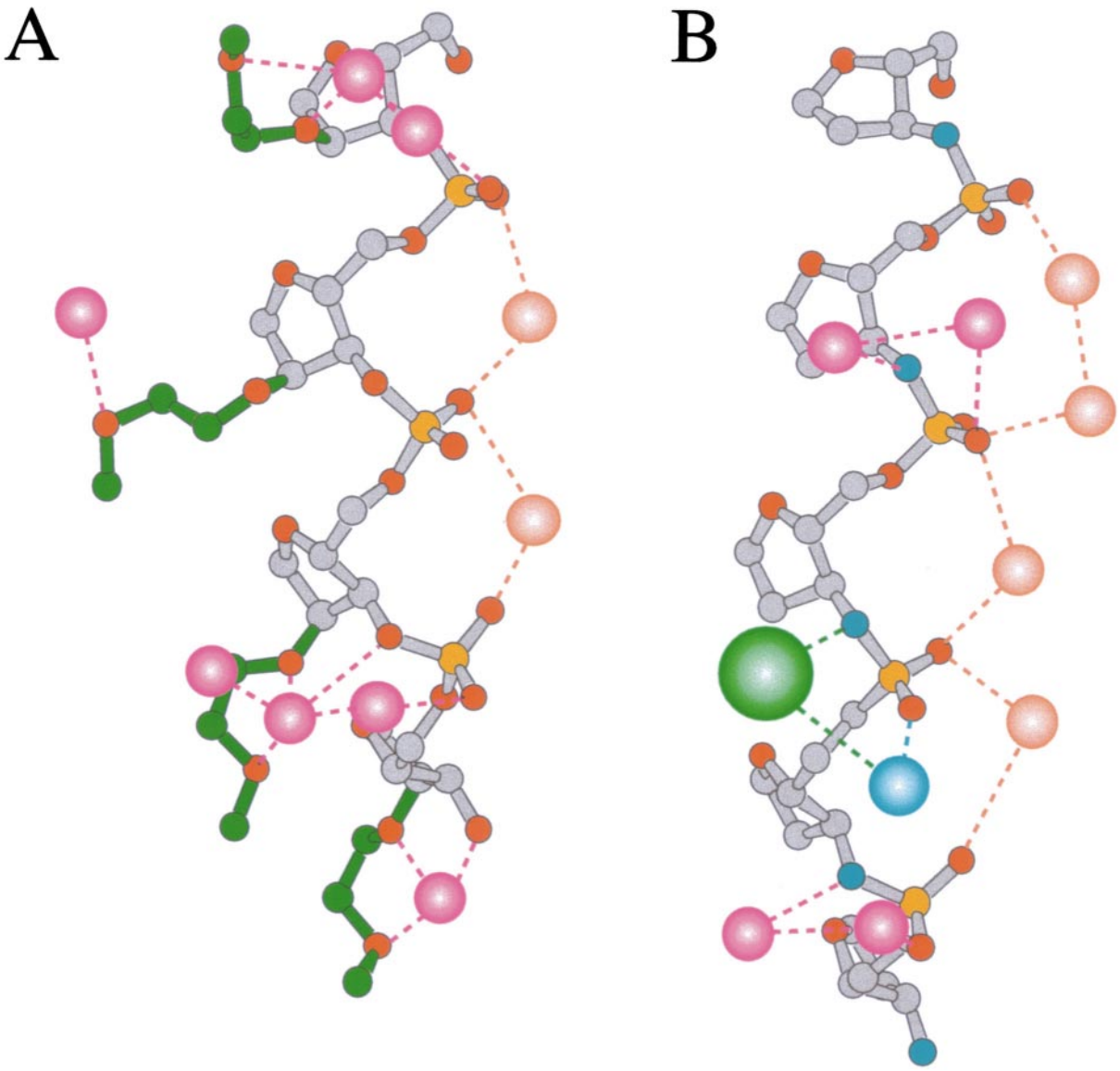

Figure 7. Hydration of the sugar-phosphate backbones in 2′-O-MOE RNA (*A*) and N3′→P5′ phosphoramidate DNA (*B*). Atoms are colored gray, red, cyan, and orange for carbon, oxygen, nitrogen, and phosphorus, respectively. Water molecules are drawn as orange (water between oxygen atoms from adjacent phosphate groups) or pink spheres [water molecules stabilized by the presence of 2′-O-MOE substituents (*A*) or 3′-nitrogens (*B*)] and hydrogen bonds are dashed lines. In the 2′-O-MOE RNA backbone portion depicted in (*A*), the 2′-O-MOE substituents (green) adopt sc^+, *ap*, sc^-, and sc^- conformations (from top to bottom). Chloride and ammonium ions replacing water molecules at several nucleotides in the crystal structure of the phosphoramidate DNA duplex are drawn as green and cyan spheres, respectively.

corners of the tetrahedron formed by 3′-amino hydrogen atoms and the fourth contact being made to an ammonium ion that in turn coordinates to three water molecules (colored in pink in fig. 8). A further ammonium ion coordinates to three phosphate groups that are located 5′ to the above amino nitrogens.

The particular arrangement of ions around the phosphoramidate DNA duplex allows unambiguous determination of the absolute configuration of the 3′-amino groups (figs 8, 9). Accordingly, the lone pair of the 3′-nitrogen is oriented *ap* to the P-O5′ bond, in good agreement with the generalized anomeric effect [see, for example, ref. 81]. In addition to the energetic advantage provided by this configuration, the lone pair and hydrogen of the 3′-amino group can engage in hydrogen-bonding interactions (fig. 9A). The corresponding

situation in P3′→N5′ phosphoramidate DNA differs drastically. To satisfy the postulated anomeric effect, the amino hydrogen is sequestered from solvent, limiting the hydration of the P3′→N5′ phosphoramidate DNA backbone (fig. 9B) [82]. Moreover, this arrangement results in steric conflicts independent of whether the 2′-deoxyribose adopts a C2′-endo or a C3′-endo conformation [48].

The crystal structure of the N3′→P5′ phosphoramidate DNA dodecamer has provided a rationalization for the enhanced thermodynamic stability of phosphoramidate DNA self-pairing relative to DNA and RNA as well as for its higher affinity for RNA. In addition, it provided an explanation for the somewhat puzzling observation that N3′→P5′ phosphoramidate DNA forms very stable duplexes, whereas P3′→N5′ phosphoramidate DNA neither pairs with itself nor RNA [42].

Structural basis of the nuclease resistance of a zwitterionic 2′-O-modified RNA

Three-dimensional structures of chemically modified nucleic acids can provide clues about the thermodynamic stabilities of their complexes with DNA and RNA. However, better understanding of the consequences of chemical modification for interactions between nucleic acid analogues and proteins requires structural models of complexes between such nucleic acids and proteins.

To gain insight into the exceptional exonuclease resistance exhibited by nucleic acids with 2′-O-(3-aminopropyl)-modified ribonucleosides (2′-O-AP RNA; fig. 1) at their 3′-flanks [26], crystal structures of complexes between short DNA fragments containing 2′-O-AP-modified residues and the DNA polymerase I Klenow fragment 3′-5′ exonuclease were determined [83]. These structures revealed that a positively charged 2′-O-AP substituent forms hydrogen bonds to an amino acid that normally coordinates to a Mg^{2+} ion at the active site of the exonuclease (fig. 10) [84]. The substituent effectively displaces this metal ion, thus preventing the stabilization of the 3′-leaving group in the second step of the phosphodiester hydrolysis reaction. The structural work demonstrates that nuclease resistance in the case of the zwitterionic RNA mimetic 2′-O-AP RNA involves an electrostatic and a steric component. The structural insights into the mechanism of exonuclease inhibition by an oligonucleotide analogue also provide a starting point for the design of new modifications with improved RNA affinity and protection against degradation by a variety of cellular nucleases.

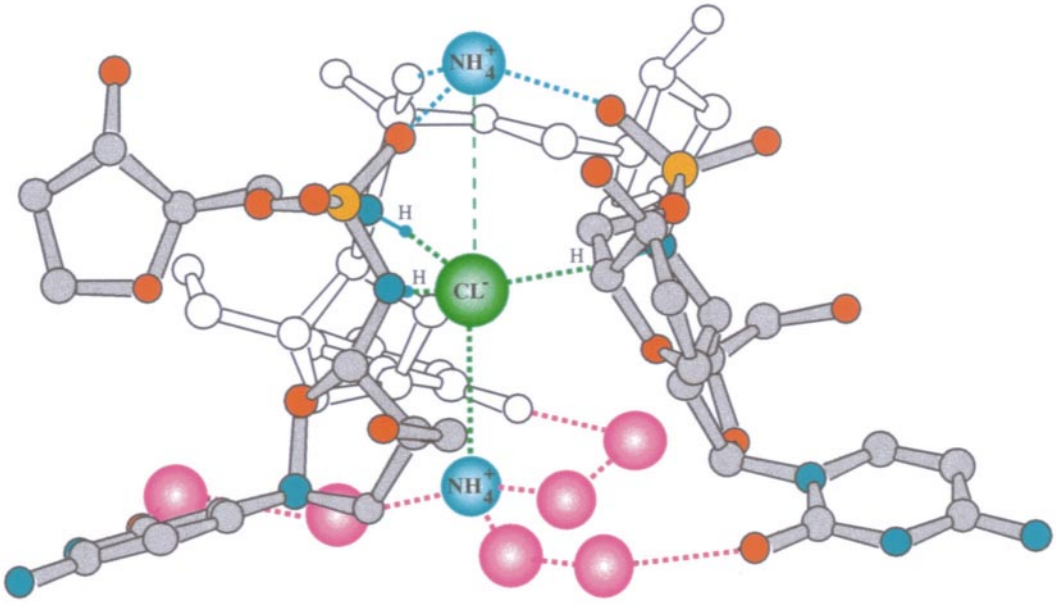

Figure 8. Bridging of three adjacent duplexes by chloride and ammonium ions in the crystal lattice of the N3′→P5′ phosphoramidate DNA dodecamer duplex. The coordination mode of the chloride anions establishes the absolute configuration of the 3′-amino nitrogen. For the two duplexes in the foreground, atoms are colored gray, red, cyan, and orange for carbon, oxygen, nitrogen, and phosphorus, respectively. Atoms of a third duplex in the background are shown in white with the 3′-nitrogen atoms highlighted in cyan. The chloride ion is drawn as a green sphere, ammonium ions are drawn as cyan spheres, hydrogen atoms of 3′-nitrogens are drawn as small cyan spheres with labels, and hydrogen bonds are dashed lines.

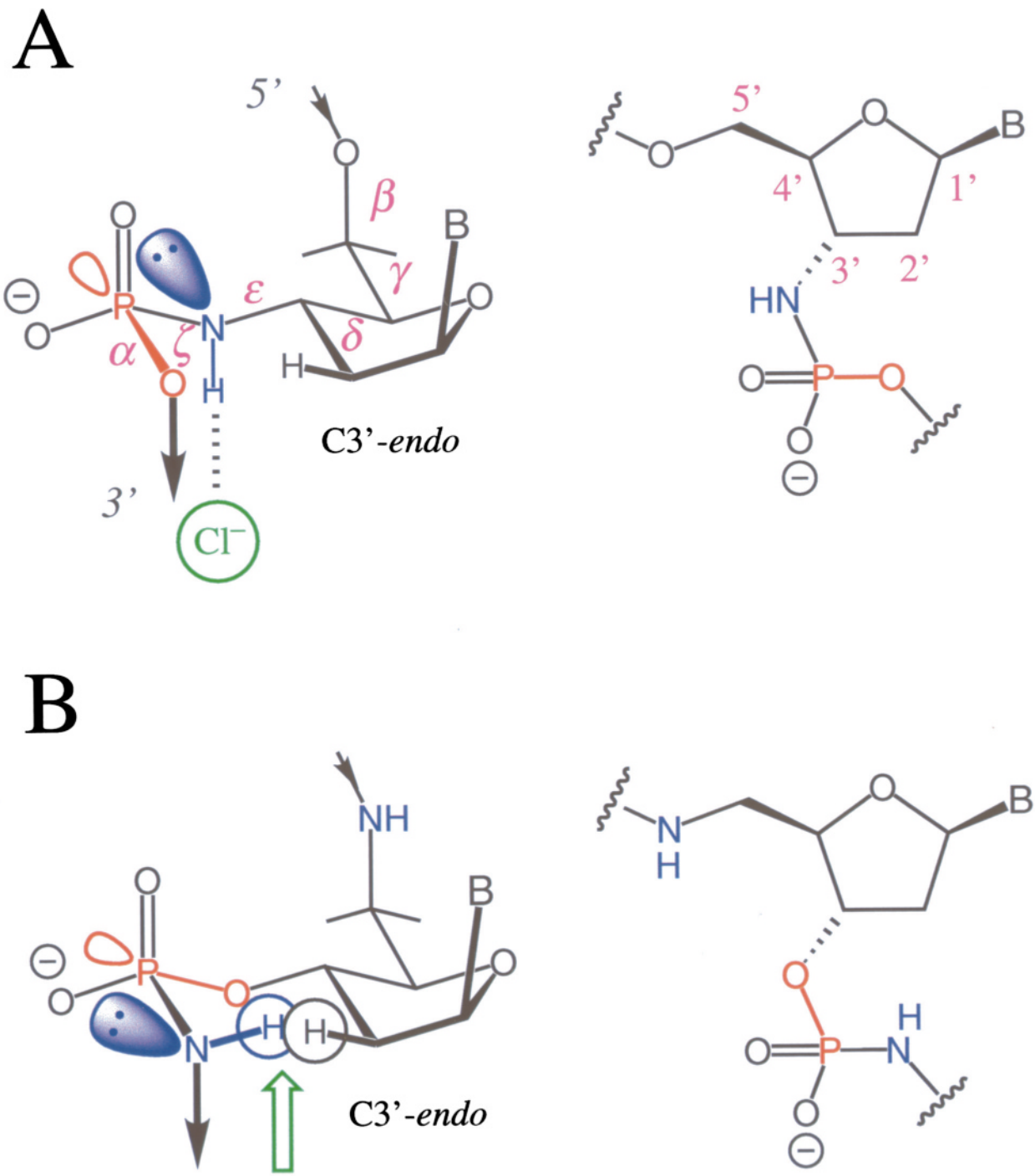

Figure 9. Structures of N3′→P5′ phosphoramidate DNA (*A*) and P3′→N5′ phosphoramidate DNA (*B*) and anomeric effects in their backbones. Coordination of chloride anions to the phosphoramidate moiety in the crystal of the N3′→P5′ phosphoramidate DNA dodecamer duplex established the *ap* orientation of 3′-nitrogen lone pair and P-O5′ bond. This experimental observation led to the prediction of an *ap* orientation between the 3′-nitrogen lone pair and P-O3′ bond in P3′→N5′ phosphoramidate DNA. The drawings show C3′-endo puckering of the deoxyribose for both types of phosphoramidates, although the presence of a 3′-oxygen in P3′→N5′ phosphoramidate DNA likely results in a preference for a C2′-endo-type pucker. However, steric conflicts are expected for P3′→N5′ phosphoramidate DNA independent of the conformation of the sugar [see fig. 9 in ref. 48]. Nitrogen lone pairs are highlighted in blue, relevant P-O bonds and antibonding orbitals are highlighted in red, a coordinated chloride anion is drawn as a green sphere, torsion angles and carbon atoms are labeled, and a predicted short contact between the 5′-amino hydrogen and one of the 2′-hydrogens is marked by an arrow.

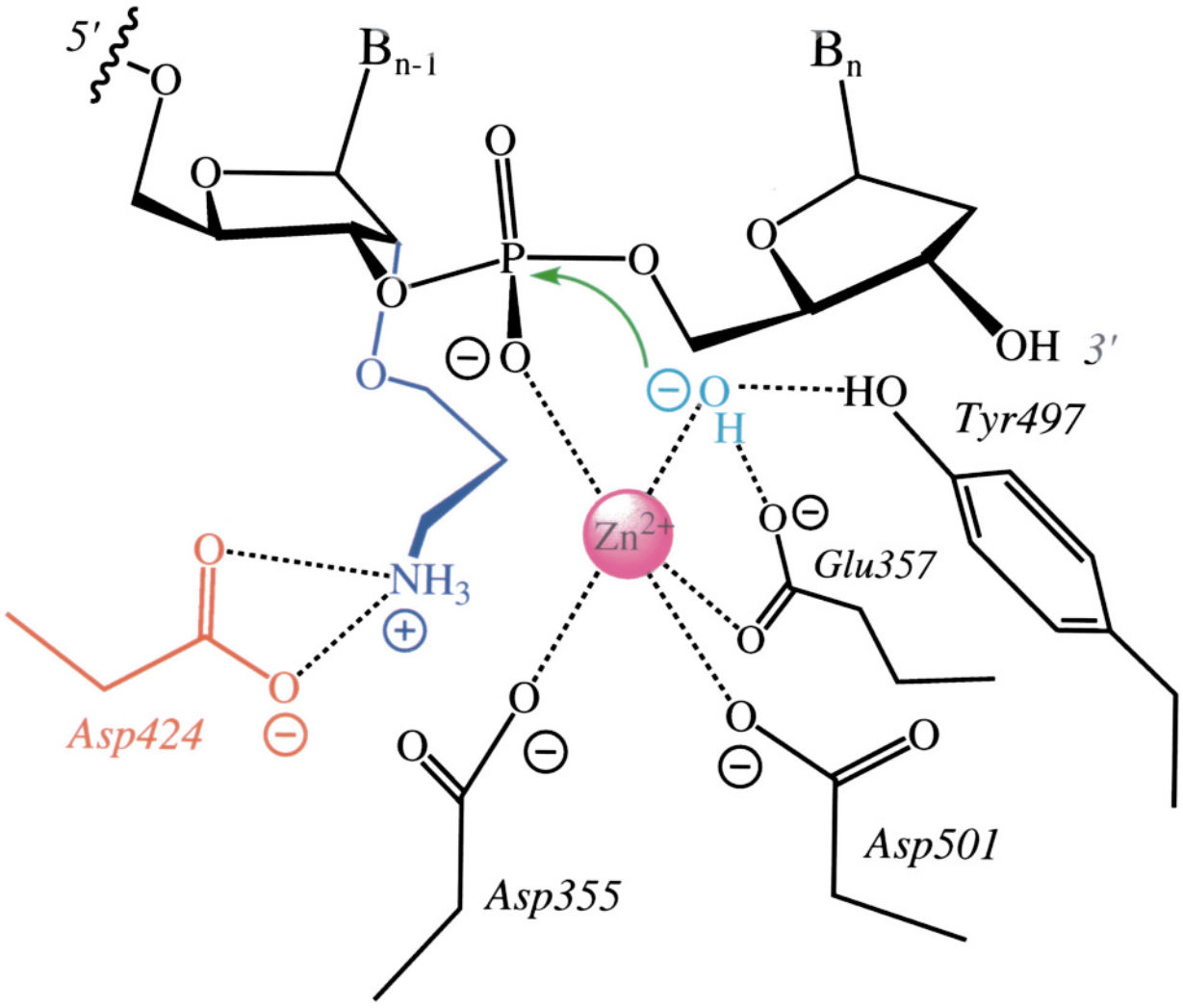

Figure 10. Model of the inhibition of DNA polymerase I Klenow fragment 3′-5′-exonuclease by an oligodeoxynucleotide carrying a 2′-O-(3-aminopropyl) (2′-O-AP) modification (blue). The model is based on X-ray crystal structures of complexes between Klenow fragment and oligodeoxynucleotides containing either a single 2′-O-AP thymine or multiple 2′-O-AP-modified residues. Presence of the positively charged aminopropyl substituent leads to displacement of metal ion B (Mg^{2+}) normally bound at the active site of the exonuclease. Rather than stabilizing this metal ion, the carboxylate of residue Asp424 (red) is engaged in hydrogen bonds to the ammonium group of the 2′-O-substituent. Metal ion A, Zn^{2+}, is shown as a pink sphere, key amino acids at the active site are labeled, the nucleophile is shown in cyan, and hydrogen bonds are dashed lines.

Conclusions

Intense efforts to define artificial nucleic acid systems with properties that render them more suitable for a host of in vitro and in vivo applications than natural DNA or RNA have now yielded a number of promising RNA analogues. These include 2′-O-modified RNAs, N3′→P5′ phosphoramidates, and LNAs. Such analogues inhibit increased RNA affinity, chemical stability, and nuclease resistance, and the DNA phosphoramidates are capable of mimicking the native RNA ligands in protein-RNA complexes. Structural investigations of 2′-O-modified RNAs and DNA phosphoramidates have provided a rationalization of the higher thermodynamic stability for these analogues. In addition to steric and stereoelectronic factors, changes in the water structure as a consequence of chemical modification appear to crucially affect thermodynamic stability. The RNA analogues reviewed here will undoubtedly find numerous applications in biochemistry and biology, and as diagnostics tools. Whether they can indeed live up to their promise as potential antisense therapeutics will have to be determined in the coming years.

Acknowledgements. We would like to thank Dr. Valentina Tereshko for help with the artwork.

1 Cech T. R., Zaug A. J. and Grabowski P. S. (1981) In vitro splicing of the ribosomal RNA precursor of *Tetrahymena*: involvement of a guanosine nucleotide in the excision of the intervening sequence. Cell **29:** 487–496

2 Guerrier-Takada C., Gardiner K., Marsh T., Pace N. and Altman S. (1983) The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. Cell **35:** 849–857

3 Doudna J. A., Couture S. and Szostak J. W. (1991) A multisubunit ribozyme that is a catalyst of and template for complementary strand RNA synthesis. Science **251:** 1605–1608

4 Doudna J. A. and Szostak J. W. (1989) RNA-catalyzed synthesis of complementary-strand RNA. Nature **339:** 519–522

5 Herschlag D. and Cech T. R. (1990) DNA cleavage catalysed by the ribozyme from *Tetrahymena*. Nature **344:** 405–409

6 Piccirilli J. A., McConnell T. S., Zaug A. J., Noller H. F. and Cech T. R. (1992) Aminoacyl esterase activity of the *Tetrahymena* ribozyme. Science **256:** 1420–1424

7 Cech T. R. (1993) Fishing for fresh catalyst. Nature **365:** 204–205

8 Unrau P. J. and Bartel D. P. (1998) RNA-catalysed nucleotide synthesis. Nature **395:** 260–263

9 Ellington A. D. and Szostak J. W. (1990) In vitro selection of RNA molecules that bind specific ligands. Nature **346:** 818–822

10 Burke D. H., Scates L., Andrews K. and Gold L. (1996) Bent pseudoknots and novel RNA inhibitors of type 1 human immunodeficiency virus (HIV-1) reverse transcriptase. J. Mol. Biol. **264:** 650–666

11 Uhlman E. and Peyman A. (1990) Antisense oligonucleotides: a new therapeutic principle. Chem. Rev. **90:** 543–584

12 De Mesmaeker A., Altman K.-H., Waldner A. and Wendeborn S. (1995) Backbone modifications in oligonucleotides and peptide nucleic acids systems. Curr. Opin. Struct. Biol. **5:** 343–355

13 Eschenmoser A. and Loewenthal E. (1992) Chemistry of potentially prebiological natural products. Chem. Soc. Rev. **21:** 1–16

14 Eschenmoser A. (1999) Chemical etiology of nucleic acid structure. Science **284:** 2118–2124

15 Singh S. K., Nielsen P., Koshkin, A. A. and Wengel J. (1998) LNA (locked nucleic acids): synthesis and high-affinity nucleic acids recognition. Chem. Commun. 455–456.

16 Koshkin A. A., Nielsen P., Meldgaard M., Rajwanshi V. K., Singh S. K. and Wengel J. (1998) LNA (locked nucleic acids): an RNA mimetic forming exceedingly stable LNA:LNA duplexes. J. Am. Chem. Soc. **120:** 13252–13253

17 Gryaznov S. and Chen J.-K. (1994) Oligodeoxyribonucleotide N3′→P5′ phosphoramidates: synthesis and hybridization properties. J. Am. Chem. Soc. **116:** 3143–3144

18 Helene C. and Toulme J.-J. (1990) Specific regulation of gene expression by antisense, sense and antigene nucleic acids. Biochim. Biophys. Acta **1049:** 99–125

19 Hall K. B. and McLaughlin L. W. (1991) Thermodynamic and structural properties of pentamer DNA · DNA, RNA · RNA, and DNA · RNA duplexes of identical sequence. Biochemistry **30:** 10606–10613

20 Egli M. (1996) Structural aspects of nucleic acid analogs and antisense oligonucleotides. Angew. Chem. Int. **35:** 1894–1909

21 Egli M., Portmann S. and Usman N. (1996) RNA hydration: a detailed look. Biochemistry **35:** 8489–8494

22 Freier S. and Altman K.-H. (1997) The ups and downs of nucleic acids duplexes stability: structure-stability studies on chemically-modified DNA:RNA duplexes. Nucleic Acids Res. **25:** 4429–4443

23 Monia B. P., Lesnik E. A., Gonzalez C., Lima W. F., McGee D., Guinosso C. J. et al. (1993) Evaluation of 2′-modified oligonucleotides containing 2′-deoxy gaps as antisense inhibitors of gene expression. J. Biol. Chem. **268:** 14514–14522

24 Altman K.-H., Fabbro D., Dean N. M., Geiger T., Monia B. P., Muller M. et al. (1996) Second-generation antisense oligonucleotides: structure-activity relationships and the design of improved signal-transduction inhibitors. Biochem. Soc. Trans. **24:** 630–637

25 Baker B. F., Lot S. S., Condon T. P., Cheng-Fluornoy S., Lesnik E. A., Sasmor H. M. et al. (1997) 2′-O-(2-methoxyethyl)-modified anti-intercellular adhesion molecule 1 (ICAM-1) oligonucleotides selectively increase the ICAM-1 mRNA level and inhibit formation of the ICAM-1 translation initiation complex in human umbilical vein endothelial cells. J. Biol. Chem. **272:** 11994–12000

26 Griffey R. H., Monia B. P., Cummins L. L., Freier S., Greig M. J., Guinosso C. J. et al. (1996) 2′-O-aminopropyl ribonucleosides: a zwitterionic modification that enhances the exonuclease resistance and biological activity of antisense oligonucleotides. J. Med. Chem. **39:** 5100–5109

27 Dean N. M., McKay R., Condon T. P. and Bennett C. F. (1984) Inhibition of protein kinase C-α expression in human A549 cells by antisense oligonucleotides inhibits induction of intracellular adhesion molecule 1 (ICAM-1) mRNA by phorbol esters. J. Biol. Chem. **269:** 16416–16422

28 Metelev V. G., Krynetskaya N. F., Purmal A. A. and Shabarova Z. A. (1990) Hybridase cleavage of RNA. I. Oligonucleotide probes for regiospecific RNA cleavage (in Russian). Bioorg. Khim. **16:** 507–513

29 Schmidt S., Niemann A., Krynetskaya N. F., Oretskaya T. S., Metelev V. G., Suchomlinov V. V. et al. (1992) The use of oligonucleotide probes containing 2′-deoxy-2′-fluoronucleosides for regiospecific cleavage of RNA by RNase H from *Escherichia coli*. Biochem. Biophys. Acta **1130:** 41–46

30 Paolella G., Sproat B. S. and Lamond A. I. (1992) Nuclease resistant ribozymes with catalytic activity. EMBO J. **11:** 1913–1919

31 Christoffersen R. E. and Marr J. J. (1995) Ribozymes as human therapeutic agents. J. Med. Chem. **38:** 2023–2037

32 Agrawal S. and Kandimalla E. R. (2000) Antisense therapeutics: is it as simple as complementary base recognition? Curr. Trends Mol. Med. Today **6:** 72–81

33 Dominski Z. and Kole R. (1993) Restoration of correct splicing in thalassemic pre-mRNA by antisense oligonucleotides. Proc. Natl. Acad. Sci. USA **90:** 8673–8677

34 Cole-Strauss A., Yoon K., Xiang Y., Byrne B. C., Rice M. C., Gryn J. et al. (1996) Correction of the mutation responsible for sickle cell anemia by an RNA-DNA oligonucleotide. Science **273:** 1386–1389

35 Alexeev V. and Yoon K. (1998) Stable and inheritable changes in genotype and phenotype of albino melanocytes induced by an RNA-DNA oligonucleotide. Nat. Biotechnol. **16:** 1343–1346

36 Kawasaki A. M., Casper M. D., Freier S. M., Lesnik E. A., Zounes M. C., Cummins L. L. et al. (1993) Uniformly modified 2′-deoxy-2′-fluoro phosphorothioate oligonucleotides as nuclease resistant antisense compounds with high affinity and specificity for RNA targets. J. Med. Chem. **36:** 831–841

37 Monia B. P., Johnston J. F., Sasmor H. and Cummins L. L. (1996) Nuclease resistance and antisense activity of modified oligonucleotides targeted to Ha-*ras*. J. Biol. Chem. **271:** 14533–14540

38 Ikehara M. (1984) 2′-Substituted 2′-deoxypurinenucleotides: their conformation and properties. Heterocycles **21:** 75–90

39 Pieken W. A., Olsen D. B., Benseler F., Aurup H. and Eckstein F. (1991) Kinetic characterization of ribonuclease resistant 2′-modified hammerhead ribozymes. Science **253:** 314–317

40 Heidenreich O., Benseler F., Fahrenholz A. and Eckstein F. (1994) High activity and stability of hammerhead ribozymes containing 2′-modified pyrimidine nucleosides and phosphorothioates. J. Biol. Chem. **269:** 2131–2138

41 Pagratis N. C., Bell C., Chang Y.-F., Jennings S., Fitzwater T., Jellinek D. et al. (1997) Potent 2′-amino- and 2′-fluoro-2′-

deoxyribonucleotide RNA inhibitors of keratinocyte growth factor. Nat. Biotechol. **15:** 68–73
42 Gryaznov S. M., Lloyd D. H., Chen J.-K., Schultz R. G., DeDionisio L. A., Ratmeyer L. et al. (1995) Oligonucleotide N3′→P5′ phosphoramidates. Proc. Natl. Acad. Sci. USA **92:** 5798–5802
43 Schultz R. G. and Gryaznov S. M. (1996) Oligo-2′-fluoro-2′-deoxynucleotide N3′→P5′ phosphoramidates: synthesis and properties. Nucleic Acids Res. **24:** 2966–2973
44 Gryaznov S. M. and Winter H. (1998) RNA mimetics: oligoribonucleotide N3′→P5′ phosphoramidates. Nucleic Acids Res. **26:** 4160–4167
45 Matray T. J. and Gryaznov S. M. (2000) Synthesis and properties of RNA analogs – oligoribonucleotide N3′→P5′ phosphoramidates. Nucleic Acids Res. **27:** 3976–3985
46 Gryaznov S. M. (1997) Synthesis and properties of the oligonucleotide N3′→P5′ phosphoramidates. Nucleosides Nucleotides **16:** 899–905
47 Chen J. K., Schultz R. G., Lloyd D. H. and Gryaznov S. M. (1995) Synthesis of oligodeoxyribonucleotide N3′→P5′ phosphoramidates. Nucleic Acids Res. **23:** 2661–2668
48 Tereshko V., Gryaznov S. and Egli M. (1998) Consequences of replacing the DNA 3′-oxygen by an amino group: high-resolution crystal structure of a fully modified N3′→P5′ phosphoramidate DNA dodecamer duplex. J. Am. Chem. Soc. **120:** 269–283
49 Ding D., Gryaznov S. M., Lloyd D. H., Chandrasekaran S., Yao S., Ratmeyer L. et al. (1996) An oligodeoxyribonucleotide N3′→P5′ phosphoramidate duplex forms an A-type helix in solution. Nucleic Acids Res. **24:** 354–360
50 Ding D., Gryaznov S. M. and Wilson W. D. (1998) NMR solution structure of the N3′→P5′ phosphoramidate duplex d(CGCGAATTCGCG) by iterative relaxation matrix approach. Biochemistry **37:** 12083–12093
51 Escude C., Giovannangeli C., Sun J.-S., Lloyd D. H., Chen J.-K., Gryaznov S. M. et al. (1996) Stable triple helices formed by oligonucleotide N3′→P5′ phosphoramidates inhibit transcription elongation. Proc. Natl. Acad. Sci. USA **93:** 4365–4369
52 Giovannangeli C., Perrouault L., Escude C., Gryaznov S. and Helene C. (1996) Efficient inhibition of transcription elongation in vitro by oligonucleotide phosphoramidates targeted to proviral HIV DNA. J. Mol. Biol. **26:** 386–398
53 Zhou-Sun B.-W., Sun J.-S., Gryaznov S. M., Liquier J., Garestier T., Helene C. et al. (1997) A physico-chemical study of triple helix formation by an oligothymidylate with N3′→P5′ phosphoramidate linkages. Nucleic Acids Res. **25:** 1782–1787
54 Roberts R. W. and Crothers D. M. (1992) Stability and properties of double and triple helices: dramatic effects of RNA or DNA backbone composition. Science **258:** 1463–1466
55 Shimizu M., Konishi A., Shimada Y., Inoue H. and Ohtsuka E. (1992) Oligo (2′-O-methyl)ribonucleosides: effective probes for duplex DNA. FEBS Lett. **302:** 155–158
56 Gryaznov S., Skorski T., Cucco C., Nieborowska-Skorska M., Chiu C.-Y., Lloyd D. et al. (1996) Oligonucleotide N3′→P5′ phosphoramidates as antisense agents. Nucleic Acids Res. **24:** 1508–1514
57 Skorski T., Perrotti D., Nieborowska-Skorska M., Gryaznov S. and Calabretta B. (1997) Antileukemia effect of c-myc N3′→P5′ phosphoramidate antisense oligonucleotides in vivo. Proc. Natl. Acad. Sci. USA **94:** 3966–3971
58 Giovannangeli C., Diviacco S., Labrousse V., Gryaznov S., Charneau P. and Helene C. (1997) Accessibility of nuclear DNA to triplex-forming oligonucleotides: the integrated HIV-1 provirus as a target. Proc. Natl. Acad. Sci. USA **94:** 79–84
59 Gryaznov S. M. (1999) Oligonucleotide N3′→P5′ phosphoramidates as potential therapeutic agents. Biochim. Biophys. Acta **1489:** 131–140
60 Rigl C. T., Lloyd D. H., Tsou D. S., Gryaznov S. M. and Wilson W. D. (1997) Structural RNA mimetics: N3′→P5′ phosphoramidate DNA analogs of HIV-1 RRE and TAR RNA form A-type helices that bind specifically to Rev and Tat-related peptides. Biochemistry **36:** 650–659
61 Saenger W. (1984) Principles of Nucleic Acid Structure, Springer, New York
62 Thibaudeau C. and Chattopadhyaya J. (1999) Stereoelectronic Effects in Nucleosides and Nucleotides and Their Structural Implications, Uppsala University Press, Uppsala
63 Egli M., Usman N. and Rich A. (1993) Conformational influence of the ribose 2′-hydroxyl group: crystal structures of DNA-RNA chimeric duplexes. Biochemistry **32:** 3221–3237
64 Cook P. D. (1991) Medicinal chemistry of antisense oligonucleotides – future opportunities. Anti-Cancer Drug Design **6:** 585–607
65 Herdewijn P. (1996) Targeting RNA with conformationally restricted oligonucleotides. Liebigs Ann. 1337–1348.
66 Cook P. D. (1998) Second generation antisense oligonucleotides: 2′-modifications. Annu. Rep. Med. Chem. **33:** 313–325
67 Herdewijn P. (1999) Conformationally restricted carbohydrate-modified nucleic acids and antisense technology. Biochim. Biophys. Acta **1489:** 167–179
68 Egli M., Tereshko V., Teplova M., Minasov G., Joachimiak A., Sanishvili R. et al. (2000) X-ray crystallographic analysis of the hydration of A- and B-form DNA at atomic resolution. Biopolymers (Nucleic Acid Sci.) **48:** 234–252
69 Nielsen C. B., Singh S. K., Wengel J. and Jacobsen J. P. (1999) The solution structure of a locked nucleic acid (LNA) hybridized to DNA. J. Biolmol. Struct. Dyn. **17:** 175–191
70 Egli M. (1998) Conformational preorganization and thermodynamic stability in oligonucleotide duplexes. Antisense Nucleic Acid Drug Dev. **8:** 123–128
71 Lubini P., Zürcher W. and Egli M. (1994) Stabilizing effects of the RNA 2′-substituent: crystal structure of an oligodeoxynucleotide duplex containing 2′-O-methylated adenosines. Chem. Biol. **1:** 39–45
72 Adamiak D. A., Milecki J., Popenda M., Adamiak R. W., Dauter Z. and Rypniewski W. R. (1997) Crystal structure of 2′-O-Me$(CGCGCG)_2$, an RNA duplex at 1.30 Å resolution: hydration pattern of 2′-O-methylated RNA. Nucleic Acids Res. **25:** 4599–4607
73 Martin P. (1995) A new access to 2′-O-alkylated ribonucleosides and properties of 2′-O-alkylated oligoribonucleotides. Helv. Chim. Acta **78:** 486–504
74 De Mesmaeker A., Häner R., Martin P. and Moser H. E. (1995) Antisense oligonucleotides. Acc. Chem. Res. **28:** 366–374
75 Altmann K.-H., Dean N. M., Fabbro D., Freier S. M., Geiger T., Häner R. et al. (1996) Second generation of antisense oligonucleotides: from nuclease resistance to biological efficacy in animals. Chimia **50:** 168–176
76 Manoharan M. (1999) 2′-Carbohydrate modifications in antisense oligonucleotide therapy: importance of conformation, configuration and conjugation. Biochim. Biophys. Acta **1489:** 117–130
77 Lesnik E. A., Guinosso C. J., Kawasaki A. M., Sasmor H., Zounes M., Cummins L. L. et al. (1993) Oligodeoxynucleotides containing 2′-O-modified adenosine: synthesis and effects on stability of DNA:RNA duplexes. Biochemistry **32:** 7832–7838
78 Tereshko V., Portmann S., Tay E., Martin P., Natt F., Altmann K.-H. et al. (1998) Structure and stability of DNA duplexes with incorporated 2′-O-modified RNA analogues. Biochemistry **37:** 10626–10634
79 Teplova M., Minasov G., Tereshko V., Inamati G. B., Cook P. D., Manoharan M. et al. (1999) Crystal structure and improved antisense properties of 2′-O-(2-methoxyethyl)-RNA. Nat. Struct. Biol. **6:** 535–539
80 Wing R., Drew H., Takano T., Broka C., Tanaka S., Itakura K. et al. (1980) Crystal structure analysis of a complete turn of B-DNA. Nature **287:** 755–758
81 Eschenmoser A. and Dobler M. (1992) Why pentose and not hexose nucleic acids? I. Helv. Chim. Acta **75:** 218–259

82 Lind K. E., Mohan V., Manoharan M. and Ferguson D. M. (1998) Structural characteristics of 2′-O-(2-methoxyethyl)-modified nucleic acids from molecular dynamics simulations. Nucleic Acids Res. **26:** 3694–3699
83 Teplova M., Wallace S. T., Minasov G., Tereshko V., Symons A., Cook P. D. et al. (1999) Structural origins of the exonuclease resistance of a zwitterionic RNA. Proc. Natl. Acad. Sci. USA **96:** 14240–14245
84 Brautigam C. A. and Steitz T. A. (1998) Structural principles for the inhibition of the 3′-5′ exonuclease activity of *Escherichia coli* DNA polymerase I by phosphorothioates. J. Mol. Biol. **277:** 363–377