Antisense Oligonucleotides

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Introduction

Until a few decades ago, molecules that exert a therapeutic effect on the human body were exclusively discovered by serendipity. As our understanding of molecular biology is increasing and we slowly gain insight on the molecular level of diseases, the rational design of drugs becomes more feasible. In particular, molecules interacting on the level of proteins were successfully designed and optimized for improved binding properties by biorational approaches. Synthetic oligonucleotides have been proposed by Zamecnik and Stephenson[1] as a new class of potential therapeutics that can interact in a rational way with the messenger RNA (mRNA) of a disease-related protein and thereby specifically inhibit its synthesis (Figure 1). The fascinating aspect of this approach, which is referred to as antisense strategy, is, on one hand, the well-understood rules of Watson−Crick base pairing by which single-stranded nucleic acids interact with complementary oligonucleotides forming a double helix (Figure 1). This predictable way of interaction represents probably one of the most powerful systems for molecular recognition designed by nature. On the other hand, every protein is synthesized by the same principal mechanism known as the central dogma of molecular biology: a gene (double-stranded DNA) carrying the genetic information for a particular protein is transcribed to single-stranded mRNA as intermediate carrier of information, which then serves as a template for the protein synthesis (translation) by ribosomes. Therefore the antisense strategy is not limited to certain types of diseases but should be widely applicable.

Nature is making use of the antisense principle as well: certain proteins are down regulated by the intracellular release of antisense RNA that is complementary to part of the target mRNA.[2] Related approaches may become important in the context of gene therapy.[3] The scope of this Account, however, is limited to short, synthetically accessible oligonucleotides or analogs thereof that are designed to interact directly with mRNA, thereby selectively inhibiting the synthesis of the target protein.

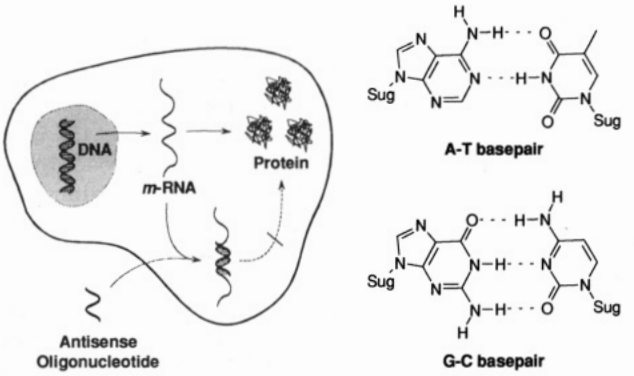

Figure 1. Schematic presentation of the antisense strategy (left) and structure of adenine−thymine (A-T) and guanine−cytosine (G-C) base pairs.

Naturally occurring oligonucleotides (DNA and RNA) do not meet the criteria for potential drug candidates, and different chemical modifications were proposed to overcome the existing hurdles,[4−10] which are briefly discussed below (Figure 2). Antisense compounds must be stable and possess a reasonable half-life *in vivo*. DNA and RNA oligonucleotides are rapidly degraded by naturally occurring nucleases that hydrolytically cleave the phosphodiester backbone. In a pharmacological sense, mRNA is the receptor that can be targeted by oligonucleotides. Therefore a high RNA binding affinity is crucial for such molecules and should directly contribute to the biological potency. Given that the sequence of the target mRNA is known, the design of potential antisense oligonucleotides should be straightforward according to the rules of base pairing. On the basis of secondary and tertiary structures and bound proteins, however, only a few binding sites are accessible on a given mRNA and these usually have to be identified by screening. The rather large and highly charged antisense molecules should be targeted to the tissue and/or organs of interest, penetrate cellular membranes, and distribute

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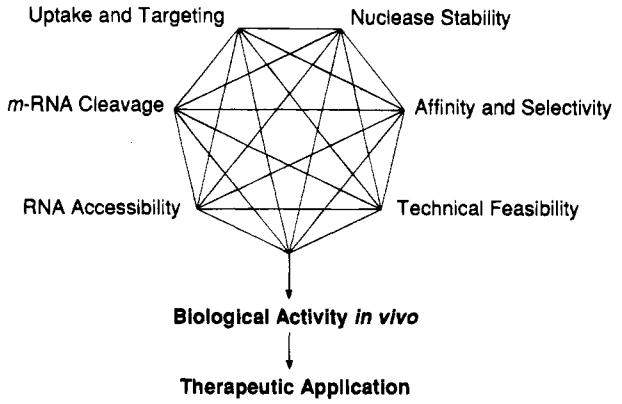

Figure 2. Major keys hurdles for the therapeutic application of antisense oligonucleotides.

intracellularly to become available at the sites of action (generation, processing, transport, or translation of mRNA). It has been demonstrated that the heteroduplex between certain antisense compounds and the target RNA is recognized by an intracellular nuclease (RNase H) that cleaves only the RNA strand of this duplex.[10] This cleavage event irreversibly destroys the mRNA and results in an increased biological efficacy. Unfortunately, this nuclease is highly sensitive to structural alterations of the antisense oligonucleotide, and therefore, most of the modifications reported to date do not support this intracellular cleavage mechanism. Finally, oligonucleotides or analogs thereof have to be prepared on a large scale for therapeutic applications. As these molecules are relatively large (molecular weight 4000–8000 Da) and so far difficult to synthesize and purify on a large scale, technical feasibility remains a major hurdle for drug development.

The focus of this Account is on our own initial chemical approaches to improve the properties of oligonucleotides, mainly concentrating on the increase of nuclease resistance and RNA binding affinity. As the latter is of high importance for the antisense strategy and usually is reflected by "melting temperatures" (T_m's), representative melting curves of modifications discussed in this Account are visualized in Figure 3. Due to the $\pi-\pi$ interaction of stacked bases in duplex nucleic acids, the UV absorption at 260 nm is quenched. Upon heating, the annealed strands unpair and become single stranded, resulting in an increased absorption at 260 nm. Ideally, an S-shaped curve is obtained from which, assuming a two-state model, the thermodynamic parameters can be calculated.[11] The midpoint of this absorption vs temperature profile is defined as T_m, reflecting the temperature at which equal fractions of oligonucleotides are paired and single stranded under equilibrium. For the oligonucleotide sequences and lengths we used in our studies, the following rule of thumb can be applied: an increase of T_m by 3–5 °C (ΔT_m) corresponds roughly to a 10-fold increase of the association constant.[12]

Melting curves or binding affinities of modified oligonucleotides were often measured against *complementary oligodeoxyribonucleotides (DNA)* which are not necessarily predictable for duplex formation with RNA.[12] For this reason, only relevant investigations

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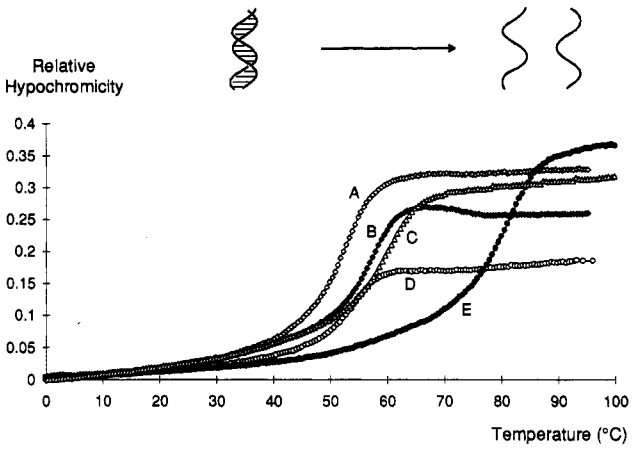

Figure 3. Representative melting curves of 5′-**tt-tt-tc-tc-tc-tc-tc**T-3′ (sequence **B** containing either 14 modified nucleosides or 7 backbone-modified dinucleosides) with the synthetic RNA complement (for details see Tables 1 and 2). A: DNA (T, C; T_m = 51.2 °C). B: DNA (T, 5-methyl-C; T_m = 56.9 °C). C: **14a** (T, C; T_m = 57.1 °C). D: **65** (T, 5-methyl-C; T_m = 52.7 °C). E: **46** (T, 5-methyl-C; T_m = 77.1 °C). The calculated difference $\Delta\Delta G°_{37}$/mod.(**46**) = 0.61 kcal (E vs B).

are cited here that dealt with RNA as a complement for hybridization studies.

Backbone Modifications

As the hydrolytic cleavage of the phosphodiester backbone is the main cause for the rapid degradation of oligonucleotides by nucleases, replacement by other moieties has been one of the major strategies to improve stability. In addition, backbone modifications can influence other properties of oligonucleotides like RNA binding affinity or behavior for cellular uptake.[10,13,14]

A first generation of backbone modifications retained the phosphorus atom as in the phosphorothioates **1**,[15] the methylphosphonates **2**,[16] the phosphoramidates **3**,[17] the phosphotriesters **4**,[18] or the phosphorodithioates **5**[19] (Figure 4). All these derivatives were shown to possess an increased resistance toward nucleases. Therefore, the corresponding oligonucleotides could be used for *in vitro* and *in vivo* experiments, but unfortunately they all displayed a lower binding affinity to the RNA target. Due to the asymmetrical substitution at phosphorus in derivatives **1**–**4**, the local plane of symmetry is destroyed

(12) The ΔT_m/mod. rather than the $\Delta\Delta G°_{37}$/mod. values (as calculated from mathematical curve fitting) from the same or similar sequences were chosen as more reliable data to generally judge the influence on binding affinity of individual modifications for fully modified oligonucleotides containing pyrimidine nucleoside analogs. For a detailed discussion of this topic, see ref 66 or the following article: Freier, S. M.; Lima, W. F.; Sanghvi, Y. S.; Vickers, T.; Zounes, M.; Cook, P. D.; Ecker, D. J. Thermodynamics of antisense oligonucleotide hybridization. In *Gene Regulation: Biology of Antisense RNA and DNA*; Erickson, R. P., Izant, J. G., Eds.; Raven Press Ltd.: New York, 1992; pp 95–107.

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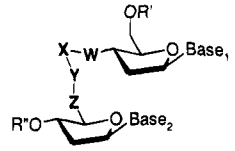
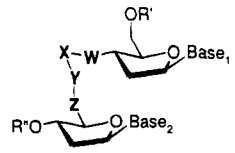


	-W-	-X-	-Y-	-Z-
1	-O-	-P(O)S$^-$-	-O-	-CH_2-
2	-O-	-P(O)CH_3-	-O-	-CH_2-
3	-O-	-P(O)NHR-	-O-	-CH_2-
4	-O-	-P(O)OR-	-O-	-CH_2-
5	-O-	-P(S)S$^-$-	-O-	-CH_2-
6	-NH-	-P(O)O$^-$-	-O-	-CH_2-

Figure 4. Structure of phosphorus-containing backbone modifications.



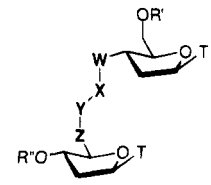
	-W-	-X-	-Y-	-Z-
7	-O-	-SiR_2-	-O-	-CH_2-
8	-CH_2-	-S-	-CH_2-	-CH_2-
9	-O-	-CH_2-	-O-	-CH_2-
10	-CH_2-	-NCH_3-	-O-	-CH_2-
11	-S-	-CH_2-	-O-	-CH_2-

Figure 5. Structure of phosphorus-free backbone modifications.

and an additional center of chirality is created. This leads, on the basis of the preparation of roughly equal amounts of both diastereoisomers during synthesis, to a large number of diastereoisomeric compounds exceeding a half-million individual compounds for a 20-mer. Nevertheless, phosphorothioates **1** can be considered as oligonucleotide analogs of the first generation and were shown to be biologically active against different molecular targets. This beneficial behavior is mainly due to biological and chemical stability, favorable pharmacokinetic properties including cellular uptake, and RNase H promoted mRNA cleavage observed for this class of compounds.[20,21] In addition to the phosphorodithioates **5**, the recently reported phosphoramidates **6** (Figure 4) represent another promising example of phosphorus-containing backbone modifications avoiding the generation of diastereoisomeric mixtures.[22] The latter analog was reported not only to add to the nuclease resistance of oligonucleotides but also to contribute to an increased RNA binding affinity.

A second generation of backbone modifications containing no phosphorus atom emerged quite recently (**7**–**11**, Figure 5).[13,14] As expected, these backbone replacements greatly increased the nuclease resistance of corresponding oligonucleotides. However, most of these modifications decrease the binding affinity to the RNA complement compared with oligodeoxyribonucleotides. Two exceptions are the *N*-methylhydroxylamine **10**[23] and the thioformacetal **11**,[24] which both displayed similar to even slightly increased RNA binding properties.

We proposed to replace the naturally occurring phosphodiester linkage by amide groups **12**–**16** (Figure 6).[25–29] The amide moiety is readily accessible by



	-W-	-X-	-Y-	-Z-
12	-NR-	-CO-	-CH_2-	-CH_2-
13	-CH_2-	-CH_2-	-NH-	-CO-
14	-CH_2-	-CO-	-NR-	-CH_2-
15	-CH_2-	-NH-	-CO-	-CH_2-
16	-CO-	-NH-	-CH_2-	-CH_2-
17	-NR_2-	-CO-	-NR_1-	-CH_2-
18	-O-	-CO-	-NR-	-CH_2-
19	-NR-	-CO-	-O-	-CH_2-
20	-CH_2-	-CO-	-CH_2-	-CH_2-
21	-CH_2-	-CH_2-	-CH_2-	-CH_2-

Figure 6. Amide, urea, carbamate, and alkyl chain containing backbone modifications.

simple and well-known synthetic methods and is compatible with the conditions required for solid phase synthesis of oligonucleotides. In addition, these linkages are achiral, thereby avoiding the problem of diastereoisomeric mixtures in oligomers. Due to their neutral character they contribute to the overall charge reduction of corresponding oligonucleotides and therefore might potentially be favorable for penetration through cellular membranes. As conformational flexibility and structural preorganization of analogs play a crucial role for hybridization properties, we investigated the behavior of the ureas **17**[30] and carbamates **18** and **19**.[31–34] In these linkages, the conjugation of the additional heteroatom with the amide bond increases the rigidity and therefore reduces the number of energetically favorable conformations. On the other hand, with the four carbon atom analogs **20** and **21**[35,36] we also studied derivatives with an increased conformational flexibility.

The average values for the variation of the melting temperatures (ΔT_m/modification) between oligonucleotides containing one to five backbone-modified T-T dimers (**tt**) **12**–**21** and the corresponding RNA complement are summarized in Table 1. These data were deduced from melting experiments of two or three different hybrid duplexes.[12]

The amide modifications **12**[37] lead to a substantial destabilization of the RNA binding affinity (ΔT_m/mod.-(**12a**) = −3.2 °C, Table 1). The exchange of the hydrogen atom by a methyl group at the amide nitrogen (**12a** → **12b**) does not significantly change the T_m of the duplex, indicating a reasonable tolerance for steric bulk at the 3′-nitrogen atom. Amides **12a** and **16**,[38] which both adopt similar geometries in the hybrid duplex with RNA, display similar T_m's. These

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Table 1. Average RNA Hybridization Data of Backbone-Modified Dinucleosides[a]

modification	ΔT_m/mod. (°C)	modification	ΔT_m/mod. (°C)
12a (R = H)	−3.2	**17b** (R_1 = CH_3, R_2 = H)	−3.5[b]
12b (R = CH_3)	−3.4	**17c** (R_1 = H, R_2 = CH_3)	−4.4[b]
13	−2.3	**18a** (R = H)	−4.1
14a (R = H)	+0.1	**18b** (R = CH_3)	−3.4
14b (R = CH_3)	−0.5[b]	**18c** (R = *i*-Pr)	−2.9
14c (R = *i*-Pr)	−0.2	**19a** (R = H)	<−4.6[b,d]
15	+0.2[c]	**19b** (R = CH_3)	<−4.5[b,d]
16	−3.7[b]	**20**	−2.7[c]
17a (R_1, R_2 = H)	<−3.6[b,d]	**21**	−5.1

[a] Small double letters indicate backbone-modified dinucleosides. If not stated otherwise, the average ΔT_m/mod. values were assembled from the following three sequences as described[66] (100 mM Na^+, 10 mM phosphate, 0.1 mM EDTA, pH 7.0 at 4 μM strand concentration and heating at 0.5 °C/min): **A**, 5′-TTT-**tt**-CTCTC-TCTCT; **C**, 5′-CTCGTAC-**tt-tt**-CCGGTCC; **D**, 5′-GCG-**tt-tt-tt-tt-tt**-GCG.[12] [b] Value determined from sequences **A** and **C**. [c] Value determined from sequences **A** and **D**. [d] **D** showed no cooperative binding to the RNA complement.

results suggest that restricted rotation near the upper sugar moiety destabilizes the duplex. If the restricted rotation around the amide bond is located near the lower sugar unit as in **13**,[27] a destabilization is observed as well. However, in this case the decrease of RNA binding affinity is not as pronounced as for **12** or **16** (ΔT_m/mod.(**13**) = −2.3 °C). This result is surprising as the corresponding torsion angle β of the amide in **13** adopts an angle close to 180° which is also observed in naturally occurring B-DNA as well as A-RNA duplexes and therefore the preorganization of this particular bond should almost be optimal. According to our molecular modeling studies,[27] amide **13** can indeed adopt a geometry close to the one of the DNA strand in a DNA/RNA hybrid. In contrast to the destabilizing backbone analogs, amide **14a**[25,26] reveals RNA binding properties similar to those of wild-type DNA (ΔT_m/mod.(**14a**) = +0.1 °C). Amides **14** and **15**[39] probably adopt similar geometries in a double helix with RNA and, as a consequence of this structural similarity, display comparable T_m values. These experimental results obtained for amides **12**–**16** suggest that the geometrical factors are predominant for the affinity behavior between a modified oligonucleotide and its RNA target.

The stability of the oligonucleotides towards endo- and exonucleases under physiological conditions was greatly increased for oligonucleotides consisting of alternating phosphodiester and amide (**14**) linkages, rendering phosphodiesters adjacent to amide backbone quite resistant toward nucleases.[25,26] Substituents are well tolerated on the nitrogen atom of amide **14** without influencing the RNA binding properties to a great extent (**14b**, **14c**) but still adding to an increased stability. According to our molecular modeling study,[26] there is enough space in the lowest energy conformation of amide **14** to accommodate a substituent as large as an isopropyl group without severe steric interactions. All our amide modifications show at least the same or even the enhanced base-pairing specificity (Watson–Crick) for the RNA complement as compared to oligodeoxyribonucleotides (DNA).

The more rigid urea derivatives **17a**–**c** (Table 1) severely disturb the ability to form heteroduplexes with RNA.[30] A minor improvement of the binding affinity compared to the ureas **17a**–**c** was observed for the carbamates **18**[31] but not for their isomers **19a** or **19b**. The introduction of extensive conformational rigidity in the backbone with a suboptimal preorganization in ureas **17** and carbamates **18** or **19** has a negative effect on the thermal stability of the corresponding duplexes. Whereas the influence of steric bulk on the 3′-nitrogen of amide **12b**, urea **17c**, and carbamate **19b** has a negligible or a small negative influence on the duplex stability, the opposite tendency is found for ureas **17b** and in particular carbamates **18b/c**, indicating the influence of additional *N*-alkyl substituents on the preferred preorganization of the carbamate moiety **18**.

The opposite of backbone rigidity was realized with the dinucleotide analogs **20** and **21** containing a flexible backbone consisting of four carbon atoms.[35,36] As expected, both analogs cause a decrease in the RNA binding affinity of the corresponding oligonucleotides, presumably due to the hydrophobic nature and the conformational restriction in the duplex structure of these flexible backbone analogs. The latter phenomenon is expected to result in an increased negative entropic contribution for the hybridization process.

In summary, the restriction of rotation has to occur preferentially in the middle of the four-atom backbone, with a preferred torsion angle of approximately 180°. Adjusting the distance between two sugar moieties and choosing the right degree of rigidity (preorganization) for the backbone are other important parameters to influence the binding behavior. With the phosphodiester replacement by amides **14** and **15** we identified two backbone analogs which not only show good binding affinity of the corresponding oligonucleotides to the RNA target but also are highly stable toward nucleolytic degradation under physiological conditions. Further synthetic modifications of these two prototypes are under current investigation to beneficially influence properties like binding affinity, cellular uptake, tissue distribution, or general pharmacokinetic properties.

A new approach to oligonucleotide analogs with a completely different backbone was realized with peptide nucleic acid (PNA) **22**.[40,41] These achiral, neutral analogs were shown to hybridize well with DNA as well as with RNA. In particular, homopyrimidine PNAs **22** bound single- and double-stranded nucleic acids by triplex formation, the latter via strand displacement.[42] When microinjected into cells, PNAs were shown to effect translational or transcriptional arrest.[43] Due to the simple synthetic accessibility of the building blocks and favorable binding behavior, PNAs seem to be promising candidates for antisense applications. However, key hurdles like cellular uptake and purification of purine-rich sequences still have to be overcome.

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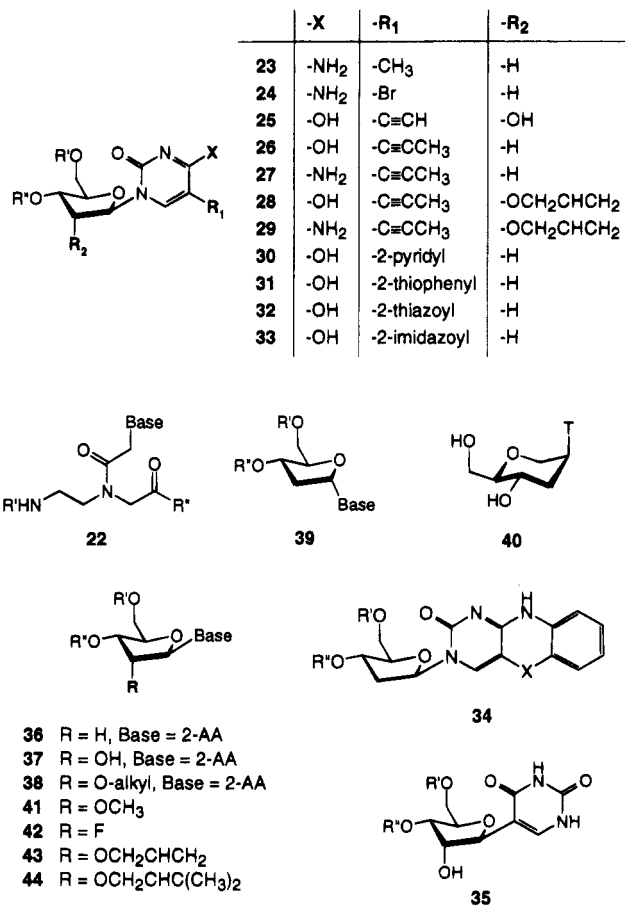

Figure 7. Modified nucleoside analogs reported in the literature that contribute to the increased RNA binding property of the corresponding oligonucleotides. 2-AA: 2-aminoadenine.

Base Modifications

The scope for modifications at the bases is rather limited as the ability of Watson–Crick base pairing should not be disrupted. Nevertheless, a few examples emerged over the past years that revealed promising properties for a potential application in antisense oligonucleotides.[44]

It was demonstrated that the substitution of cytosines using 5-methyl- or 5-bromocytosines (**23** and **24**, Figure 7) in oligodeoxyribonucleotides increased the stability of DNA/RNA hybrids.[45] A similar behavior was found for thymine replacing uracil in different types of nucleoside analogs, indicating beneficial hydrophobic interactions of the C(5)-methyl groups in duplex structures (data not shown; see also Figure 3). However, C(5)-substituents with longer alkyl chains significantly reduce the stability of DNA duplexes,[46] even though longer aminoalkyl groups might behave differently.[47]

The introduction of triple bonds at C(5) of pyrimidine bases dramatically influences the binding behavior: the replacement of uridine by 5-ethynyluridine **25** in poly(rU) resulted in a high-affinity increase to poly(rA).[48] Oligonucleotides containing 5-(1-propynyl)-2′-deoxyuridine **26** and -cytidine **27** significantly enhance the RNA binding affinity,[49] probably due to additional $\pi-\pi$ stacking in the duplex between the additional C(5)-substituent and the adjacent base on the 5′-side. The corresponding hybrid duplex was shown to be accepted by RNase H, resulting in cleavage of the bound RNA. As the influence on the nuclease resistance is only marginal, the backbone was stabilized by phosphorothioate linkages yielding oligonucleotides which were shown to be potent inhibitors of gene expression when microinjected in cells or delivered with cell-permeabilizing agents.[50] The combination of the latter base modification with the 2′-*O*-allyl substituent yielded the nucleoside analogs **28** and **29** that greatly increase the RNA binding affinity and nuclease resistance of the corresponding oligonucleotides.[51] Quite recently, the scope of substituents at C(5) was extended to heteroaromatic groups (**30**–**34**) which were shown to beneficially influence the stability of RNA duplexes.[52] Thiazole derivative **32** (ΔT_m/mod. = +1.7 °C) increased the thermal stability comparable to the propyne U **26** (ΔT_m/mod. = +1.6 °C).[52a] However, the most dramatic influence was reported for the phenoxazine **34** (X = O) showing a dramatic stabilization of duplexes (depending on neighboring sequences) between 2 and 5 °C per modification.[52b]

Pseudouridine ψ (**35**) is a structural isomer of uridine in which the heterocyclic base is linked to the ribose sugar by the carbon atom. With the replacement of a N-nucleoside by a C-nucleoside, two imino hydrogens rather than one are present in pseudouridine. Incorporation of **35** in oligonucleotides was shown to stabilize the hybrid duplex with natural RNA (ΔT_m/mod. = +1.8 °C).[53]

2,6-Diaminopurines **36**–**38** as adenine analogs can form an additional hydrogen bond when base pairing to uracil or thymine. Hybridization studies revealed that substitutions of adenosines in DNA or RNA oligonucleotides using 2-aminoadenines (2-AA) stabilize duplexes with DNA and especially RNA complements.[54–56] Due to an increased lability for depurination, especially the more stable ribo derivatives **37** and **38** are useful for a potential application in antisense oligonucleotides. Short probes containing 2-amino-2′-*O*-alkyladenosines **38** allowed the efficient binding and separation of complementary RNA in HeLa splicing extracts.[57]

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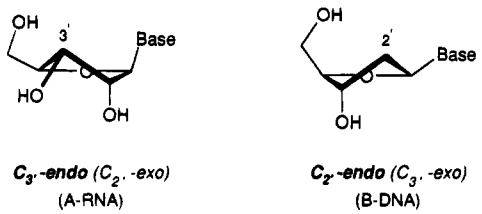

$C_{3'}$-*endo* (C_2,-*exo*) (A-RNA) $C_{2'}$-*endo* (C_3,-*exo*) (B-DNA)

Figure 8. Two main families of sugar conformations found in A-RNA (left) and B-DNA (right).

Sugar Modifications

A positive change in T_m was found for oligonucleotides having incorporated the α-anomeric nucleosides **39** in place of the natural β-anomers (parallel orientation of the strands in the α-DNA/β-RNA hybrid).[58] The high nuclease stabiity of this type of oligonucleotides was experimentally proven; in fact they are not substrates for nucleases.[59] It was shown that α-oligodeoxyribonucleotides are useful as inhibitors of translation in cell lysates.[60]

According to a publication dealing with the investigation of various hexapyranosyl-like oligonucleotides, a tridecanucleotide of nucleoside **40** exhibited excellent binding characteristics toward poly(rA) (ΔT_m/mod. = +1.0 °C) thereby representing the first hexapyranosyl-derived oligonucleotide analog with excellent RNA binding properties.[61]

Besides a small structural variation of the pyrimidine bases (in DNA thymine is replacing uracil), the 2′-substituent on the furanose ring is the primary chemical differnce between DNA and RNA. It has been shown that electronegative substituents like fluorine or oxygen (RNA derivatives) stabilize the $C_{3'}$-*endo* conformation,[62] probably due to a preferred gauche orientation of the 2′-substituent and the ring oxygen (Figure 8). As a consequence RNA is found predominantly in the $C_{3'}$-*endo* conformation that is exclusively present in the A-form duplexes.[63] Short RNA/RNA duplexes are usually much more stable than the DNA/DNA duplex of the same sequence.[64] We therefore chose as our working hypothesis to mimic RNA-like or $C_{3'}$-*endo*-like structures with nucleoside analogs in order to beneficially influence the RNA binding behavior.

Effects of 2′-substituents on hybrid stability were reported: the incorporation of 2′-*O*-methyl ribonucleosides **41**[65,66] or 2′-fluoro-2′-deoxyribonucleosides **42**[67] in oligonucleotides increases the affinity toward the RNA complement. Even though the 2′-*O*-methyl derivatives are more resistant against degradation by nucleases than DNA, both classes of oligonucleotides are not stable enough for antisense applications. However, the replacement of the phosphodiester linkages by phosphorothioates yielded oligonucleotides that not only were highly resistant but retained good binding affinity to the RNA targets as well.[67]

Uniform incorporation of 2′-*O*-allyl ribonucleosides **43**[66] also stabilized hybrid duplexes, whereas the substitution with 2′-*O*-(3,3-dimethylallyl) ribonucleosides **44** destroyed the ability of an oligonucleotide to bind its complementary RNA sequence, indicating the intolerance of steric bulk in the corresponding duplexes.[68]

A clear structure–activity relationship between duplex stability and the size of the 2′-*O*-alkyl substituent was reported.[66] Compared to 2′-*O*-methyl ribonucleotides **41**, the destabilizing effect on RNA binding ability increases with the size of 2′-*O* substituents: the average ΔT_m per modification correlates well with the number of carbon atoms in the chain. As expected, the larger groups at the 2′-position add to the nuclease resistance of oligonucleotides. From this point of view, 2′-*O*-alkyl ribonucleosides linked with phosphodiester groups were not too attractive for an antisense application as the increased nuclease resistance goes along with a decrease of duplex stability.

With this background, we surprisingly found that 2′-*O*-alkoxy substituents, in particular 2′-methoxyethoxy, 2′-methoxytriethoxy, and groups related to ethylene glycol, retain or even slightly surpass the high RNA binding affinity of 2′-*O*-methyl ribonucleosides **41** despite the large substituents in the 2′-position (Table 2).[69] The rationale for this behavior can be hypothesized in two ways: first, by additional solvation of the alkoxy substituent in water. However, this influence on the duplex stability is difficult to predict. Nevertheless binding results of 2′-modified carbocyclic nucleoside analogs suggest that a 2′-methoxytriethoxy substituent, as compared to the corresponding 2′-methoxy group, has a rather negative influence on the RNA duplex formation (*vide infra*, Table 4, **60** and **61**). On the other hand, the second oxygen atom in the 2′-chain of the ethylene glycol type ribonucleosides **46**, **47**, and **49–51** is responsible for a structural preorganization of the side chain which, due to the *gauche* effect, preferably adopts a conformation with a translation angle 2′-*O*–CH_2–CH_2–O of either +60° or −60°. For all the listed derivatives, both conformations are well accommodated in the minor groove of the formed hybrid duplex without causing any steric constraints. In addition, model building indicated that additional side groups on the second carbon atom of the 2′-substituent would fit extremely well, irrespective of the new stereocenter formed as was proven with derivatives **50** and **51** in a preliminary hybridization experiment (Table 2). This

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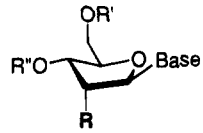
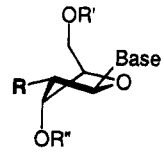
Table 2. Average RNA Hybridization and Stability Data of 2′-*O*-Alkyl Nucleosides[a]



	R	ΔT_m/mod. (°C)	stab.[h]
41	OCH_3	+1.0	8
45	$OCH_2CH_2CH_3$	+0.8	
46	$OCH_2CH_2OCH_3$	+1.1	24
47	$O(CH_2CH_2O)_3CH_3$	+1.1	>48
48	$OCH_2OCH_2CH_3$	−0.3	16
49	(*R*)-$OCH_2CH(OCH_3)CH_3$	+1.6[e]	
50	(*R*)-$OCH_2CH(OH)CH_2OH$	+1.5[f]	
51	(*S*)-$OCH_2CH(OH)CH_2OH$	+1.5[f]	
52	$OCH(CH_3)_2$	−0.4[c]	
53	OC_6H_5	<−1.2[b,d]	11

[a] The average ΔT_m/mod. values were assembled from two or three sequences as described[66] (**A**, 5′-TTTTtCTCTCTCTCT; **B**, 5′-**ttttt**c^m**tc**^{m}**tc**^{m}**tc**^{m}**tc**^{m}T (**c**^{m}: C(5)-methyl derivatives); **C**, 5′-CTCG-TAC**ttttt**CCGGTCC; **D**, 5′-GCG**ttttttttttt**GCG; small letters stand for modified nucleotide analogs).[12] If not stated otherwise, the values given are based on sequences **A**, **C**, and **D**. [b] Value determined from sequences **A** and **C**. [c] Value determined from sequences **A** and **D**. [d] **D** showed no cooperative binding to the RNA complement. [e] Value determined from sequences **A** and **B**. [f] Value based on a single modification incorporated in **A**. [g] Value based on uracil instead of thymine. [h] The factor of increased stability in 10% heat denatured calf fetal serum was established as compared to the wild type DNA oligonucleotide. The half-life was determined for the sum of n and $(n-1)$ species of 5′-TCCAGGTGTC-CG**ttt**C.

Table 3. Average RNA Binding and Stability Data of 2′-Alkyl Nucleosides[a]



	R	ΔT_m/mod. (°C)	stab.[h]
54	CH_3	<−2.3[b,d]	>48
55	CH_2CHCH_2	<−3.8[b,d]	
56	(*E*)-$CHCHC_6H_5$	<−4.5[b,d,g]	>32
57	C_6H_5	<−3.7[b,d]	>48
58	CH_2OH	<−3.0[b,d]	3

[a] For details, see Table 2, footnotes *b*–*h*.

hypothesis is even further supported with the ribonucleoside analog **48** which prefers, due to the anomeric effect, a different and unfavorable conformation of the side chain resulting in a slightly destabilizing contribution for the duplex formation. As expected, the increasing size of the 2′-chain adds to the nucleolytic stability, yielding promising nucleoside analogs for antisense oligonucleotides with derivatives **46** and **47**.

The importance of electronegative substituents in 2′-position is best demonstrated with derivatives **54**–**58** carrying alkyl, alkenyl, or aryl side chains (Table 3).[70] Whereas the nucleolytic degradation of the corresponding oligonucleotides is greatly inhibited, all nucleoside analogs examined revealed a strong negative impact on duplex formation, probably due to a predominant $C_{2'}$-*endo* sugar conformation together

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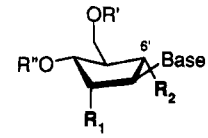
Table 4. Average RNA Binding and Stability Data of Carbocyclic Nucleosides[a]



	R_1, R_2	ΔT_m/mod. (°C)	stab.[h]
59	H, H	+0.3[i]	2.5
60	OCH_3, H	−0.9	29
61	$O(CH_2CH_2O)_3CH_3$, H	<−2.0[b,d]	>48
62	H, OH	+0.5	7
63	H, OCH_3	−0.9	10
64	H, $O(CH_2)_4NH_2$	−1.8[c]	>24
65	H, CH_3	−0.2	15

[a] For details, see Table 2, footnotes *b*–*h*. [i] This average value was taken from different sequences.[74] A ΔT_m/mod. of +0.4 °C was determined for 5′-**ttttttctctctctc**T against the RNA complement under the same experimental conditions.

with additional steric constraints between the 2′-methylene group and the adjacent phosphodiester linkage. Even the incorporation of electronegative atoms in the β-position as in **58** did not alter this behavior.

Another approach was realized by exchanging the oxygen atom of the furanose ring with a methylene group, giving carbocyclic nucleoside analogs. While oligonucleotides containing carbocyclic 2′-deoxyribonucleotides were reported,[71–74] substituted derivatives were largely unexplored so far. The good RNA binding affinity but insufficient nuclease resistance of carbocyclic 2′-deoxyribonucleotides **59** urged us to search for candidates with improved stability.[74] On the basis of model considerations we focused on derivatives carrying additional substituents in either the 2′- or 6′-position.[75] Opposite to 2′-*O*-methyl ribonucleosides **41** (Table 2), the corresponding carbocycle **60** (Table 4) slightly destabilizes the RNA hybrid duplexes, illustrating the importance of the *gauche* effect in ribonucleosides between the ring oxygen and 2′-oxygen to stabilize the $C_{3'}$-*endo* sugar conformation. Of the substituents examined in the 6′-position, only the hydroxy derivative **62** was able to enhance the RNA binding affinity of oligonucleotides. Larger substituents as in **64** beneficially influence the nuclease resistance but increasingly destabilize the hybrid duplexes.

Oligonucleotide Conjugates

Substitution with relatively simple molecules can result in a profound effect on the properties of oligonucleotides. Consequently, oligonucleotides have been equipped with a large number of chemical groups such as intercalators, hydrophobic residues, alkylating groups, or other chemically reactive molecules. The

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purpose of such oligonucleotide modifications is the improvement of antisense properties like increased binding affinity, nuclease resistance, and enhanced cellular uptake. Attachment of the residues can be achieved through 3′- or 5′-termini as well as through sugar moieties, nucleobases, or internucleotidic linkages. Introduction of the residues can be carried out postsynthetically, most commonly by reaction with a free amino or sulfhydryl group previously introduced into the oligonucleotide. Alternatively, substituents can also be incorporated during oligonucleotide synthesis by using the corresponding phosphoramidites or other suitable building blocks. Since the synthesis of oligonucleotide conjugates has been the topic of recent reviews,[5,76,77] we will concentrate on the most important classes of conjugates and their impact on antisense properties of oligonucleotides, thereby citing only selected publications.

Intercalating Groups. Attachment of intercalating groups greatly increases the affinity of an oligonucleotide toward a complementary strand. Stabilization of the duplex is a result of $\pi-\pi$ interactions between the intercalator and the nucleobases. The most commonly used intercalators are derivatives of acridine,[78] anthraquinone,[79,80] and pyrene.[81] A considerable increase in T_m, typically in the range of 5−10 °C per intercalating residue, is observed for an average-sized oligonucleotide. In certain cases, however, even larger effects have been reported.[78,82] The contribution for the increased binding affinity is dependent not only on the nature of the intercalator but also on the site of attachment to the oligonucleotide[79,83] as well as on the linker.[80,84]

Hydrophobic Groups. Such residues have been linked to oligonucleotides, primarily to improve cellular uptake. Within this class of conjugates, cholesterol-modified oligonucleotides have been investigated most intensively. Substitution with a cholesterol moiety has a pronounced effect on cellular uptake.[85−88] It was shown that uptake of such conjugates proceeds via an endocytotic pathway.[85,88] Detailed studies, though, revealed that a major part of these derivatives remains encapsulated inside stable endosome-like particles and therefore is unable to bind the target mRNA.[88] Upon substitution of antisense oligonucleotides with cholesterol,[86] cholic acid,[89] or long aliphatic alkyl chains,[90,91] increased antiviral efficiency was observed in cellular assays. On the other hand, substitution with cholesterol or a lipid resulted also in nonspecific, sequence-independent effects.[91,92] Lipophilic molecules have been attached to oligonucleotides using linkers which can be cleaved by intracellular enzymes.[86,93] Liberation of the oligonucleotide by action of intracellular enzymatic activity was demonstrated in the case of disulfide-[93] or ester-linked[86] hydrophobic residues.

Chemically Reactive Groups. Oligonucleotides bearing chemically reactive groups can be used for sequence specific modification and/or cleavage of targeted nucleic acids.[94] *In vitro* translation of rabbit globin mRNA was substantially reduced after incubation with an antisense oligonucleotide bearing an alkylating group.[95] The extent of inhibition correlated well with the amount of cross-linking. Photoinduced cross-linking to complementary single-stranded oligonucleotides was described with aryl azide,[96] porphine,[97] and psoralene[98,99] derivatized oligonucleotides. After hybridization, cross-linking with psoralene conjugates was effected by irradiation at 350 nm.[98] Cross-linking could be reversed by irradiation at 254 nm. Psoralene-modified oligonucleotide methylphosphonates were specifically cross-linked to single-stranded regions of rabbit globin mRNA. This treatment led to a considerable reduction of *in vitro* translation.[99]

Metal complexes were covalently attached to oligonucleotides for the specific cleavage of nucleic acids, a field which has been comprehensively reviewed.[100,101] Among the best investigated compounds are iron and copper complexes. In particular, single-stranded DNA[100] but also RNA[102−104] were cleaved by such conjugates at specific sites in a metal-dependent redox reaction. Lanthanides and complexes thereof were found to cleave RNA by a hydrolytic pathway,[105] probably acting at the same time as acid and base catalysts. The corresponding oligonucleotide conjugates were recently reported to act as artificial ribonucleases, albeit with rather low efficiencies.[106−108] Using novel europium complexes covalently linked to

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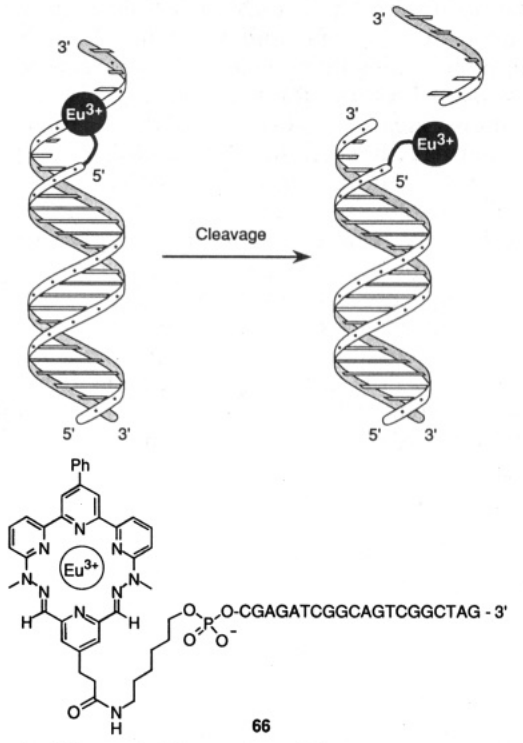

Figure 9. Schematic illustration of the sequence-specific RNA cleavage by oligonucleotide conjugate **66**.

an oligodeoxynucleotide (**66**), we achieved an almost quantitative cleavage (88%) of synthetic, complementary RNA at physiological pH and temperature (Figure 9).[109] Due to the high chemical stability of these complexes this approach might become useful to enhance the potency of modified antisense compounds. A different approach was realized with conjugates that are hybrid molecules between oligonucleotides and nucleases or nuclease fragments.[110–112]

Poly(L-lysine). Due to the known ability of poly(L-lysine) to promote cellular uptake of drugs,[113] oligonucleotides have been conjugated to this basic polypeptide[114,115] and recently also to poly(L-ornithine).[116] Poly(L-lysine) conjugates showed specific activity against vesicular stomatitis virus (VSV) in cultured cells at concentrations as low as 100 nM.[115] In other assays, however, these conjugates also induced nonspecific effects.

It is worthwhile mentioning that often several beneficial effects are observed upon substitution of oligonucleotides with a single chemical group; e.g., attachment of intercalators, lipophilic groups, or poly(L-lysine) not only influences binding properties or cellular uptake but generally also results in increased nuclease resistance.

Outlook

Even though phosphorothioates are generally believed to represent the first generation of antisense oligonucleotides, they suffer from certain drawbacks like the existence of diastereoisomeric mixtures, intermediate RNA binding affinity, and certain nonspecific side effects.[21] The work presented and cited above clearly indicates that solutions for antisense oligonucleotides with improved binding properties and nuclease resistance can be realized by various chemical approaches. In addition, novel achievements were presented which can influence the biological efficacy by modulating the biodistribution and/or stability of the target mRNA by cleavage. However, if these new promising candidates will be useful as a second generation of antisense compounds remains to be proven by their *in vivo* pharmacokinetic and pharmacodynamic behavior. So far, the collection of *in vivo* data is limited exclusively to methylphosphonates and in particular phosphorothioates which have entered the clinical trials as the first generation of antisense compounds.

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