Chemically Modified Antisense Oligonucleotides—Recent Improvements of RNA Binding and Ribonuclease H Recruitment

Oliver Seitz*

Early this year the first oligonucleotide drug, the phosphorothioate ISIS-2922 (Fomivirsen), against CMV retinitis was admitted to the market. Twenty-one years after the introduction of the antisense approach this new drug might serve as the ultimate proof of principle.[1] In the antisense concept an exogeneously added antisense oligomer selectively binds to the target mRNA and blocks the translation. A great deal of the attractiveness of this approach arises from the possibility of designing RNA binders according to the well-known Watson–Crick base-pairing rules. Thus, with such designed binders it would be feasible to virtually knock out any (pathogenic) protein at will. Phosphorothioates, the first generation of antisense oligomers, exhibit a relatively low binding affinity to the target RNA. However, the increased cellular uptake and nuclease resistance render them biological active. In addition, ribonuclease H (RNase H), an enzyme that cleaves RNA in DNA · RNA hybrids, accepts phosphorothioate · RNA hybrids and allows for the destruction of multiple copies of mRNA per antisense oligomer. Rather than providing a comprehensive overview of the progress in antisense technology, this highlight focuses on recent improvements of key elements such as RNA binding and RNase H recruitment.

Flanagan and co-workers reported on an impressive enhancement of the potency of an antisense phosphorothioate (S-AON).[2] Their 20-mer unit with only one base modification inhibited the expression of the *c-raf* gene at nanomolar concentrations. This translated into a 25 fold increase of the antisense potency relative to a previously optimized 20-mer S-AON, which is currently being tested in phase II clinical trials for the treatment of cancer. The modified heterocycle is based on a cytosine analogue synthesized by Lin and Matteucci.[3] A tricyclic phenoxazine **2** serves as a rigid scaffold for the attachment of groups suitable for further interactions with nucleobases (Figure 1). By appending an aminoethyloxy tether, the phenoxazine was armed with a strong hydrogen bond donor that would recognize both the Watson–Crick and the Hoogsteen sites of guanine, and was thus termed a G-clamp (**3**; Figure 2). The cytosine analogues

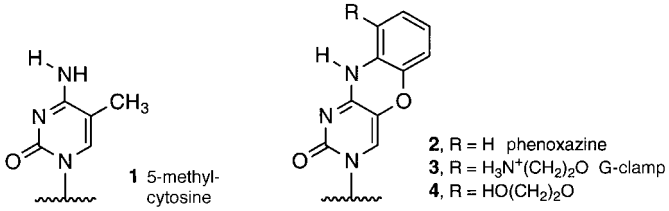

Figure 1. Cytosine analogues used in hybridization experiments (Table 1) by Lin and Matteucci.[3]

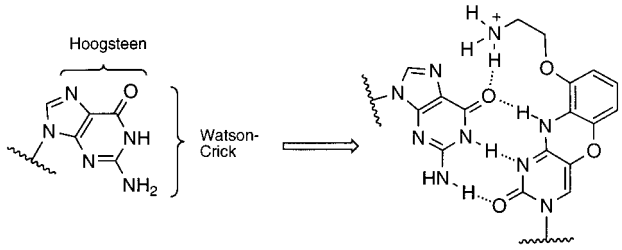

Figure 2. Proposed interaction of the G-clamp with the Watson–Crick and the Hoogsteen sites of guanosine.

shown in Figure 1 were incorporated into antisense oligonucleotides (AON) and hybridized to the complementary oligodeoxynucleotide (ODN). The melting temperature T_M, a measure of the thermodynamic stability of double stranded ODNs, were determined by analysis of the temperature-dependent UV absorbance. The G-clamp-containing AON **3** displayed a dramatically enhanced stability (Table 1), while

Table 1. Analysis of the melting temperatures of different single phenoxazine-modified AONs hybridized to matched and single-mismatched ODNs.[a]

	T_M [°C] (ΔT_M [°C])			
X	**Y** = G[b]	**Y** = A[c]	**Y** = T[c]	**Y** = C[c]
1 (5-Me-C)	50.5	32.0 (−18.5)	30.0 (−20.5)	29.0 (−21.5)
2 (phenoxazine)	57.0 (+6.5)	44.5 (−12.5)	42.0 (−15.0)	33.0 (−24.0)
3 (G-clamp)	68.5 (+18.0)	45.5 (−23.0)	41.0 (−27.5)	40.0 (−28.5)
4	51.5 (+1.0)	–	–	–

[a] Buffer: 0.14 M KCl, 5 mM Na_2HPO_4, 1 mM $MgCl_2$, pH 7.2. Test AON: 5′-TCTC**X**CTCTC-3′. Target ODN: 3′-AGAG**Y**GAGAGA$_5$-5′. [b] Matched ODNs: ΔT_M relative to **X** = **1**. [c] Mismatched ODNs: ΔT_M relative to **Y** = G.

the AON with the weakly hydrogen bonding hydroxyl group (phenoxazine **4**) replacing the amino group in **3** showed a very similar T_M to the parent 5-methylcytosine **1**. In order to rule out any nonspecific effects that might be caused by ionic interactions, the authors studied the dependence of the T_M on the salt concentration. Attractive Coulomb interactions would normally be weakened at high ionic strength. However, the increased affinity of the G-clamp-modified AONs relative to the 5-methylcytosine was independent of the salt concentration ($\Delta T_M = 16–18$ °C in the range of 0.014–1.4 M KCl), which provided further support for the proposed specific hydrogen binding. Most importantly, the G-clamp AON conferred an enhanced mismatch specificity relative to

[*] Dr. Oliver Seitz
Institut für Organische Chemie der Universität
Richard-Willstätter-Allee 2, D-76128 Karlsruhe (Germany)
Fax: (+49) 721-608-4825
E-mail: seitz@ochhades.chemie.uni-karlsruhe.de

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5-methylcytosine as determined by the comparison of the T_M values of matched versus mismatched hybridization (Table 1).

The greatly increased affinity and specificity of the base-modified G-clamp was confirmed in recent in vivo studies.[2] African green monkey kidney cells (CV-1 cells) were transfected with a plasmid that contained the human cyclin-dependent kinase inhibitor $p27^{kip1}$ and treated with a cytofectin and the antisense phosphorothioate (S-AON). The G-clamp (S-AON) **9** (Table 2) inhibited $p27^{kip1}$ expression by

Table 2. RNase H activity of the posphorothioates **7**, **8**, and **9**.

Compound	Sequence	Base modifications	RNase H cleavage [%][a]
7	5′-TGG CTC TCC TGC GCC-3′	none	21.7
8	5′-**5**GG **656 566 5**G**6** G**66**-3′	5-propinyluracil/cytosine	4.9
9	5′-TGG CTC TC**3** TGC GCC-3′	G-clamp	17.4

[a] The % cleavage of complementary radioactively labeled RNA 5 min after treatment with nuclear extracts of HeLa cells (source of human RNase H).

96 % at 30 nM, as measured by Western Blotting of cellular extracts. At this concentration the previously most potent S-AON **8**, which contains 11 C5-propinyl-modified bases (**5** and **6** in Figure 3) showed 67 % inhibition of $p27^{kip1}$ levels. A comparison of the IC_{50} values revealed that the mono substituted strand **9** was three times more potent than the

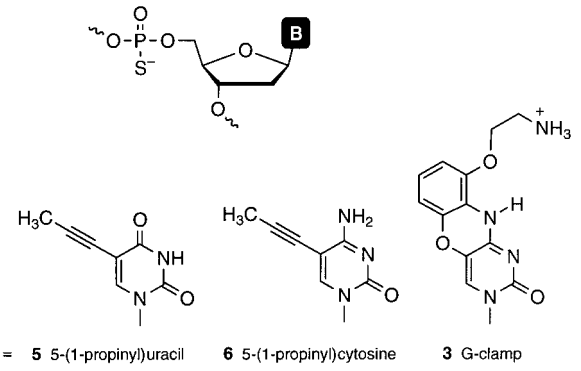

Figure 3. Nucleobase analogues incorporated in antisense agents (Table 2) by Flanagan and co-workers.[2]

multiply substituted strand **8**. The control S-AON **7** failed to demonstrate any inhibitory activity under these conditions. The sequence specificity of the G-clamp S-AON **9** was tested by transfecting the CV-1 cells with the wild-type p27 plasmid or a single base pair mutant. This mutation changed the complementary guanine in the transcribed mRNA to a uracil group. The G-clamp S-AON **9** inhibited the expression of the single base pair mutant $p27^{kip1}$ with a fivefold decrease in the IC_{50} value relative to the wild-type $p27^{kip1}$. In contrast, the inhibition of wild-type and mutant $p27^{kip1}$ was almost identical when the C5-propinyl-substituted S-AON **8** was used. The increased antisense activity of the G-clamp S-AON **9** was even more obvious when gel-shift assays were performed. The less potent C5-propinyl-substituted S-AON **8** had an affinity for the RNA target that was almost twice as high as **9**. Thus, differential induction of RNase H was expected to play an important role. The RNase H catalyzed cleavage assay demonstrated that the G-clamp S-AON · RNA heteroduplex was degraded 3.5 times more rapidly than the C5-propinyl substituted S-AON · RNA heteroduplex, and almost as rapidly as the unmodified duplex with **7** (Table 2). These results indicate that RNase H activation appears to be of major importance for antisense activity.

Nearly all nuclease-resistant antisense oligonucleotides currently in clinical trials contain modified phosphodiester backbones. However, the alteration of the natural D-2-deoxyribose itself can also provide AONs with increased stability against nuclease degradation. Unfortunately, none of the uniformly sugar-modified AONs were able to induce mRNA cleavage by RNase H catalysis. Damha and co-workers reported on a class of RNase H competent AONs based on arabinose, the 2-epimer of ribose.[4] It was demonstrated that substituting the 2′-OH group of the arabinonucleic acids (ANA) with fluorine yielded 2′-deoxy-2′-fluoro-β-D-arabinonucleic acids (2′F-ANA) with a markedly enhanced affinity towards complementary RNA (Figure 4). For example, the melting temperatures of the RNA heteroduplexes with 2′F-ANA **10** and **14** were significantly increased relative

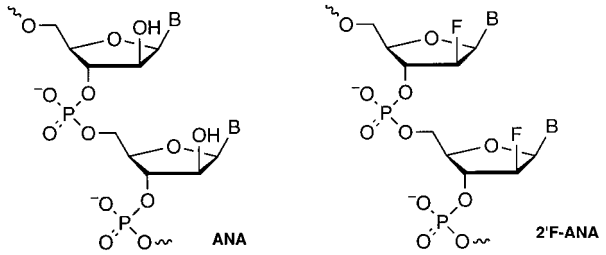

Figure 4. Chemical structure of arabino nucleic acids (ANA) and 2′-deoxy-2′-fluoroarabinonucleic acids (2′F-ANA). (B = nucleobase).

to the hybrids formed with the ANAs **11** and **15** or with the DNA strands **12** and **16** (Table 3). The higher T_M value of the 2′F-ANA · RNA heteroduplexes with **10** and **14** relative to the corresponding hybrids with the phosphorothioates (S-AON) **13** and **17** is also worth mentioning. In addition, the circular dichroism (CD) spectra of the ANA · RNA and 2′F-ANA · RNA duplexes closely resembled that of the parent DNA · RNA duplex, which suggests the common existence of an A-type helix. The specific recognition of the A-like helix of

Table 3. Analysis of the melting temperatures of the arabinonucleic acid duplexes with RNA and control DNA · RNA and S-ODN · RNA duplexes.[a]

AON sequence	Backbone modification	T_M [°C]
5′-TTT TTT TTT TTT TTT TTT TTT-3′	2′F-ANA, **10**	44
	ANA, **11**	[b]
	DNA, **12**	39
	S-DNA, **13**	21
5′-TTA TAT TTT TTC TTT CCC-3′	2′F-ANA, **14**	65
	ANA,[c] **15**	32
	DNA, **16**	51
	S-DNA, **17**	38

[a] Buffer: 0.14 M KCl, 5 mM Na_2HPO_4, 1 M $MgCl_2$, pH 7.2. [b] Not detectable. [c] Contained uracil instead of thymine.

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DNA · RNA duplexes by RNase H is assumed to induce the selective hydrolysis of DNA · RNA duplexes even in the presence of RNA · RNA or DNA · DNA duplexes. In fact, similarly to the AON **12** and the S-AON **13**, the 2′F-ANA **10** was able to induce a RNase H catalyzed cleavage of the radioactively labeled RNA target. Neither the duplexes of the epimeric 2′-deoxy-2′-fluororibonucleic acids (2′F-RNA) nor the corresponding duplexes with RNA or RNA alone were attacked. Interestingly, ANA · RNA duplexes were poor substrates for RNase H, a result that was attributed to the low stability of the duplex. The 2′F-ANAs are the first class of uniformly sugar-modified antisense oligonucleotides that display an enhanced affinity for their RNA target and fully maintain the ability to induce RNase H. However, it has to be mentioned that this criterion is also met by antisense oligomers consisting of a non-uniform backbone. For example, chimera of DNA and peptide nucleic acids (PNA) are also substrates for RNase H.[5]

Although the importance of RNase H induction had been emphasized, it is still a matter of debate as to whether high affinity binding to the mRNA alone might be sufficient enough to inhibit translation. A marked increase of the RNA binding affinity and the nuclease resistance would be of pivotal significance. Remarkably, stable pairing with RNA was reported for modifications with altered internucleotide linkages such as 3′-thioformacetal,[6] methylene(methylimino) (MMI),[7] and methylene amide spacers.[8] Similiarly, sugar-modified oligomers such as 2′-O-alkylribonucleosides,[9] hexitolnucleosides,[10] or α-nucleosides[11] confer high-affinity RNA binding. Successful examples in which the entire ribose phosphate backbone has been replaced by an artificial backbone include morpholinophosphorodiamidates[12] and peptide nucleic acids.[13] An alternative approach, introduced by Leumann et al., makes use of rigidified ODN analogues.[14] The restricted conformational flexibility eventually reduces the pairing entropy ΔS and this translates into a more favorable standard enthalpy ΔG of duplex formation. In this regard, several base-pairing systems were reported.[15] The most recent example of a conformationally restricted Watson – Crick base-pairing system was published by Wengel and co-workers, who introduced the locked nucleic acids (LNA; Figure 5).[16] The methylene bridge that connects the 2′-oxygen with the 4′-carbon atom locks the sugar in the C-3′-endo conformation typical of the A-type RNA · RNA duplex,

Figure 5. Chemical structure of locked nucleic acids (LNA) and proposed locked sugar moiety in the puckered C-3′-*endo* conformation.

as demonstrated by the CD spectrum of a LNA · RNA duplex.[17] The hybridization of the mixed-sequence all-LNA nonamer **19** with the complementary DNA nonamer yielded a duplex with an unprecedented thermodynamic stability. The T_M value was increased by 36 °C relative to the corresponding DNA · DNA duplex with **18** (Table 4).[18] The **19** · RNA hybrid displayed an even higher increase of the thermal stability (ΔT_M of 46 °C) relative to the DNA · RNA duplex. The introduction of a single base pair mismatch resulted in a decrease of the T_M value by 12 – 14 °C, a satisfactory selectivity that accounts for the very high binding affinity of LNA for the DNA or RNA targets. To date, LNA · LNA hybridization constitutes the most thermally stable nucleic acid type duplex system as demonstrated by the T_M of 93 °C for the duplex of **19** with its complementary LNA strand.[17]

Table 4. Analysis of the melting temperatures of LNA duplexes with DNA, RNA, and LNA as well as control DNA duplexes with DNA and RNA.[a]

Compound	AON sequence	AON type	Complementary ODN	T_M[°C] (ΔT_M[°C])
18	5′-d(GTG ATA TGC)-3′	DNA	DNA	28 (–)
			RNA	28 (–)
19	5′-GTG ATA TG^{Me}C-3′	LNA	DNA	64 (36)
			RNA	74 (46)
			LNA	93[b] (> 65)

[a] Buffer: 0.1 M NaCl, 10 mM Na_2HPO_4, pH 7.0. [b] A low salt buffer (1 mM Na_2HPO_4, pH 7.0) had to be used since duplex dissociation was not detectable in the standard buffer.

The examples presented above indicate that key elements of antisense technology such as RNA binding affinity and selectivity, RNase H induction as well as nuclease resistance can be improved. The employment of the various strategies in concert certainly holds much promise for the development of a new generation of antisense agents, demonstrated best by the successful combination of the phosphorothioate methodology with the modified G-clamp base.

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