Pure & Appl. Chem., Vol. 69, No. 3, pp. 437–440, 1997.
Printed in Great Britain.

Backbone modifications for antisense oligonucleotides

Alain De Mesmaeker*, Adrian Waldner, Sebastian Wendeborn, Romain M. Wolf

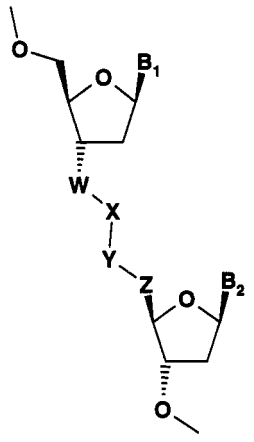
Central Research Laboratories, Ciba-Geigy Ltd., CH-4002 Basel, Switzerland

Abstract: Eighteen different types of backbone replacements have been investigated. The thermodynamic stability of the duplexes formed between these modified oligonucleotides and their RNA complements are summarized. Among these backbone replacements, we identified two amide derivatives which display improved properties fort their application to the antisense strategy. Some important structural and conformational elements which contribute to the thermodynamic stability of the duplexes were deduced from the melting temperatures (T_m's) data and from molecular modeling and ^{1}H-NMR studies.

Antisense oligodeoxyribonucleotides constitute a promising class of potential new therapeutical agents that would interact selectively and in a predictable manner with a target RNA (ref. 1). This interaction would occur by specific *Watson-Crick* base pairing between the antisense and the complementary RNA strand. The formation of the duplex would prevent the expression of a defined disease-related protein. However, the naturally occurring oligonucleotides (DNA and RNA) do not meet the criteria for potential drug candidates. Chemical modifications of the 2'-oligodeoxyribonucleotides are required to improve several of their properties. Among them, the resistance of antisense oligonucleotides towards cellular nucleases has to be substantially increased, keeping (or ideally improving) the affinity and the specificity for the complementary RNA target. The poor penetration of the 2'-oligodeoxynucleotides into cells has also to be improved. Therefore, various replacements of the naturally occurring phosphodiester backbone, which is responsible for the instability of the oligodeoxynucleotides in vivo and, at least partially, for their low ability to cross cell membranes, were recently investigated (ref. 1). Unfortunately, most of the replacements of the phosphodiester linkage leading to an increased resistance towards nucleases are also connected to a decrease in affinity for the RNA complement. So far, only very few examples of backbone replacements which are not disfavoring the formation of a duplex with RNA were reported (ref. 2, 5, 6).

We summarize here our results on the backbone modifications that we incorporated into 2'-oligodeoxynucleotides (Figure 1). Initially, we proposed to replace the natural phosphodiester group by an amide as in **6** - **10**, keeping four atoms between two sugar moieties (ref. 3-7). The main reasons for this choice were the novelty of the structures, the compatibility of the amide bond with the conditions for the solid phase synthesis of oligonucleotides. An amide bond should be more stable under physiological conditions than a phosphodiester bond. The overall charge reduction of the oligonucleotides containing neutral amide bonds should favor their penetration through cell membranes. The amide moiety is readily accessible by simple synthetic methods and is achiral, thereby avoiding mixtures of diastereomers in oligodeoxyribonucleotides. Moreover, our molecular modeling studies suggested that these amide modified backbones would be compatible with a duplex structure with an RNA strand.

These amide modifications **6** - **10** were compared to the more rigid or more flexible backbone replacements (Figure 1) in terms of thermodynamic stability of the duplexes formed with an RNA complement. In most cases, the melting temperature (T_m) of 3 duplexes (Sequences A - E, Table 1) were measured. In Table 2 the average values for the variation of the melting temperature of the duplexes (ΔT_m/modification) are summarized.



	W	X	Y	Z
1	O	$PO(O^-)$	O	CH_2
2	NR_1	CO	NR_2	CH_2
3	O	CO	NR	CH_2
4	NR	CO	O	CH_2
5	S	CO	NR	CH_2
6	NR	CO	CH_2	CH_2
7	CO	NR	CH_2	CH_2
8	CH_2	CH_2	NR	CO
9	CH_2	CO	NR	CH_2
10	CH_2	NR	CO	CH_2
11	CH_2CH_2	CO	NH	CH_2
12	CH_2	--	NH	CO
13	CO	NH	--	CH_2
14	CH_2	CH =	CH	CH_2
15	CH_2	CO	CH_2	CH_2
16	CH_2	CH_2	CH_2	CH_2
17	O	CH_2	C ≡	C
18	S	CH_2	C ≡	C
19	CH_2	CH(OMe)	C ≡	C

Fig.1 Natural and modified backbones in oligonucleotides

TABLE 1. Oligomers prepared (5' → 3')

Entry	Sequences	T_m (^oC) Wild type with RNA complement
A	CTC GTA CCT*TTC CGG TCC	63.3
B	CTC GTA CT*T T*TC CGG TCC	61.8
C	GCG T*T T*T T*T T*T T*T GCG	50.2
D	TTT T*TC TCT CTC TCT	52.8
E	T*T T*T T*C T*C T*C T*C T*CT	52.8

* = Backbone modification

The incorporation of all the backbone replacements **2** - **19** into oligonucleotides was realized using the corresponding dimers carrying at the 5'-end a dimethoxytrityl group and at the 3'-end a phosphoramidite group. The replacement of the phosphodiester bond by rather rigid backbones as ureas **2**, carbamates **3**, **4** and thiocarbamates **5** lead to a severe depression of the melting temperature of the duplexes (Table 2, entry 1). Due to electronic delocalization of the lone pairs of the heteroatoms into the carbonyl group, these backbones are too rigid and cannot adopt a favorable conformation compatible with a stable duplex structure. These modifications **2** - **5** are not useful for an antisense application.

The five isomeric amide backbones **6** - **10** were incorporated into oligonucleotides. Using molecular mechanics, we could not determine which isomer would be the most favorable. However, our molecular dynamics studies (ref. 15) suggested qualitatively that the amides **9**, **10**, having the restricted rotation in the middle of the backbone would be more appropriate for their incorporation into a duplex with RNA. This was fully confirmed experimentally. The amide modifications **6** - **8** lead to a decrease of the melting temperature (Table 2, entry 2). An increase of the size of the substituent on the nitrogen atom of the amide **6** had a negative effect on the stability of the duplex. Interestingly, the amides **6** and **7**, which are two analogs of the same C=C bond furnished very similar T_m values (ΔT_m/mod. = -2.8°C for **6** R = H; ΔT_m/mod. = -3.5°C for **7** R = H).

In contrast, a slight increase of the melting temperature was observed for the amide modification **9** where R = H (ΔT_m/mod. = +0.4°C). In an alternating backbone as in sequence E (Table 1), the increase in T_m per amide unit **9** was +0.6°C. A similar trend was observed for the amide **10** which adopts the same conformation as the amide **9** (ref. 15). In the case of the modification **10**, an increase in T_m was also reached when several units were incorporated in an alternating mode with phosphodiesters (ΔT_m/mod. = +0.4°C for **10** in sequence C). In order to determine the importance of the trans geometry in the middle of

the backbone, we incorporated the trans C=C bond **14**, as well as its cis isomer into oligonucleotides. Our molecular modeling studies predicted that both geometries would be compatible with a duplex structure. Indeed, we obtained very similar T_m values for the cis and for the trans olefins (Table 2, entries 12, 13). The decrease in T_m observed for the trans olefin compared to the amides **9** and **10** could be attributed to disfavorable hydrophobic interactions between the olefinic backbone and water. Although the geometrical factors seem to be predominant, the ability of an amide (R = H) to form hydrogen bonds with water molecules contribute to the overall stabilization of the system. In agreement with this assumption, the introduction of hydrophobic residues on the nitrogen atom of the amide **9** (Table 2, entries 4 -7) causes a decrease of the melting temperature of the duplex. The substitution of the phenyl ring (Table 2, entry 6) with a p-octyl chain (Table 2, entry 7) lowered substantially the T_m (ΔT_m/mod. = -0.7°C for **9** R = phenyl, ΔT_m/mod. = -3.5°C for **9** R = p-octyl-phenyl). These results suggest that in addition to the conformation of the backbone, interactions of the substituents with the aqueous phase should be taken into consideration. The overall length of the amide backbone is also critical. The additional -CH_2- residue in the backbone **11** leads to a lower T_m than for the prototype **9** (ΔT_m/mod. = -1.8°C for **11**). The removal of a methylene group also leads to a decrease of the T_m, especially in the case of the modification **13** (Table 2, entry 11, ΔT_m/mod. = -5.0°C). The importance of the preorganization of the backbone reached with the amides **9**, **10** and with the olefin **14** was further demonstrated by the severe drop of T_m observed for the more flexible backbones **15** and **16**.

TABLE 2. Average ΔT_m values per backbone modification

Entry	Modification	Substituents	ΔT_m/modification (°C)	References
1	**2 - 5**	H, Me, Et, i-Pr	-2.0 - -5.7	8 - 10
2	**6 - 8**	H, Me, i-Pr	-1.6 - -3.6	3,4,7
3	**9**	H	+0.4	5
4	**9**	Me	-0.1	5
5	**9**	i-Pr	0.0	5
6	**9**	Ph	-0.7	11
7	**9**	p-(n-Octyl)-phenyl	-3.5	11
8	**10**	H	0.0	6
9	**11**	H	-1.8	11
10	**12**	H	-1.8	11
11	**13**	H	-5.0	11
12	**14**	cis	-1.2	12
13	**14**	trans	-0.8	12
14	**15**		-2.5	13
15	**16**		-4.2	13
16	**17**		-2.4	14
17	**18**		-4.6	14
18	**19**	R	-2.0	14
19	**19**	S	-2.7	14

Finally, we incorporated the acetylenic modifications **17** - **19** into oligonucleotides. A severe depression of the T_m was observed for the modifications **17** and **18** where an oxygen or a sulfur atom was linked to the 3'-carbon of the upper sugar unit. However, the backbone modification **19** where the sugar residues are linked over four carbon atoms could adopt a conformation compatible with a duplex structure (Table 2, entries 18, 19). As a single replacement of the phosphodiester bond (sequence D), the modification **19** R led to a severe destabilization of the duplex (ΔT_m/mod. = -3.6°C). Interestingly, the same modification **19** R led only to a small depression of the T_m of the duplex (ΔT_m/mod. = -0.5°C) when introduced in an alternating mode with phosphodiester moiety (sequence C). In contrast, the **19** S epimer showed pronounced destabilization in both sequences (ΔT_m/mod. = -3.1°C in sequence D; ΔT_m/mod. = -2.3°C in sequence C).

In conclusion, we identified two backbone modifications **9** and **10**, which have a favorable effect on the thermodynamic stability of the duplex with RNA. Recently, we focused on the modification **9**, due to its better synthetic accessibility compared to amide **10**, allowing the preparation of sufficient amounts of

oligonucleotides for their biological evaluation in vitro and in vivo. The alternating phosphodiester-amide backbone **9** confers to the antisense oligonucleotide not only a good affinity for an RNA target but also a highly increased stability under physiological conditions. The phosphodiester bond located between two amide **9** residues is no longer a good substrate for endo- and exonucleases. Recently, we established that the oligonucleotides having an alternating phosphodiester-amide **9** backbone are sufficiently stable in cells to allow their specific interaction with a mRNA, resulting in the inhibition of the translation of a target protein. Further synthetic modifications of the very promising amide **9** backbone replacement, which might be required to reach ultimately potent biological activity, are under current investigation.

REFERENCES

1. a) P.C. Zamecnik, M.L. Stephenson. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 280; b) E. Uhlmann, A. Peyman. Chem. Rev. **1990**, *90*, 543; c) S.T. Crook. *Annu. Rev. Pharmacol. Toxicol.* **1993**, *32*, 329; d) A. De Mesmaeker, R. Häner, P. Martin, H.E. Moser. *Acc. Chem. Res.* **1995**, *28*, 366; e) A. De Mesmaeker, K.-H. Altmann, A. Waldner, S. Wendeborn. *Curr. Opin. Struct. Biol.* **1995**, *5*, 343.
2. a) J.J. Vasseur, F. Debart, Y.S. Sanghvi, P.D. Cook. *J. Amer. Chem. Soc.* **1992**, *114*, 4006; b) R.J. Jones, K.Y. Lin, J.F. Milligan, S. Wadwani, M.D. Matteucci. *J. Org. Chem.* **1993**, *58*, 2983; c) S. Gryaznov, J.K. Chen. *J. Amer. Chem. Soc.* **1994**, *116*, 3143; d) L. De Napoli, A, Iadonisi, D. Montesarchio, M. Varra, G. Piccialli *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1647.
3. J. Lebreton, A. De Mesmaeker, A. Waldner, V. Fritsch, R.M. Wolf, S.M. Freier. *Tetrahedron Lett.* **1993**, *34*, 6383.
4. A. De Mesmaeker, J. Lebreton, A. Waldner, V. Fritsch, R.M. Wolf, S.M. Freier. *Synlett* **1993**, 733.
5. a) A. De Mesmaeker, A. Waldner, J. Lebreton, P. Hoffmann, V. Fritsch, R.M. Wolf, S.M. Freier. *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 226; b) J. Lebreton, A. Waldner, C. Lesueur, A. De Mesmaeker. *Synlett* **1994**, 137. See also: I. Idziak, G. Just, M.J. Damha, P.A. Giannaris. *Tetrahedron Lett.* **1993**, *34*, 5417.
6. J. Lebreton, A. Waldner, V. Fritsch, R.M. Wolf, A. De Mesmaeker. *Tetrahedron Lett.* **1994**, *35*, 5225.
7. A. De Mesmaeker, J. Lebreton, A. Waldner, V. Fritsch, R.M. Wolf. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 873.
8. A. Waldner, A. De Mesmaeker, J. Lebreton, V. Fritsch, R.M. Wolf. *Synlett* **1994**, 57.
9. A. Waldner, A. De Mesmaeker, J. Lebreton. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 405; See also: I. Habus, J. Temsamani, S. Agrawal. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1065.
10. A. Waldner, A. De Mesmaeker. *Synlett*, **1995**, 108.
11. A. De Mesmaeker, A. Waldner, S. Wendeborn, R. M. Wolf, unpublished results. See also: G. Stork, C. Zhang, S. Gryaznov, R. Schultz *Tetrahedron Lett.* **1995**, *36*, 6387.
12. S. Wendeborn, R.M. Wolf, A. De Mesmaeker. *Tetrahedron Lett.* **1995**, *36*, 6879.
13. A. De Mesmaeker, A. Waldner, Y.S. Sanghvi, J. Lebreton. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 395. See also: C.V.C. Prasad, T.J. Caulfield, C.P. Prouty, A.K. Saha, W.C. Schairer, A. Yawman, D.A. Upson, L.I. Kruse *Bioorg. Med. Chem. Lett.* **1995**, *5*, 411.
14. S. Wendeborn, C. Jouanno, R. M. Wolf, A. De Mesmaeker. *Tetrahedron Lett.*, **1996**, *37*, 5511.
15. V. Fritsch, A. De Mesmaeker, A. Waldner, J. Lebreton, M.J.J. Blommers, R.M. Wolf. *Bioorg. Med. Chem.* **1995**, *3*, 321.