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Short communication

Nuclease stability as dominant factor in the antiviral activity of oligonucleotides directed against HSV-1 IE110

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Abstract

The anti-herpes simplex virus type 1 (anti-HSV-1) efficacy of a series of oligonucleotides was determined as a function of their chemical structure. All oligonucleotides consisted of the same sequence directed against the translation initiation codon of the HSV-1 immediate early gene. The oligonucleotides were modified with phosphorothioate linkage patterns according to various protection strategies against nucleolytic degradation. We show that nuclease resistance is the dominant factor that determines the antiviral efficacy in this series. A minimal protection strategy, consisting of end-capping and pyrimidine protection, has proven to be particularly useful, because it not only yields nuclease-resistant oligonucleotides but also minimizes non-sequence-specific effects. Copyright © 1997 Elsevier Science B.V.

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Antisense oligonucleotides have been shown to act as specific inhibitors of gene expression in a variety of in vitro and in vivo systems (Uhlmann and Peyman, 1990; Field and Goodchild, 1995). The major problems that are met with antisense oligonucleotides are their rapid degradation by various nucleolytic activities within cells or in serum and their poor cellular uptake. Since the internucleotide phosphodiester bond is the site of the nucleolytic attack, many modifications of this linkage have been reported to improve nuclease stability, the most popular one still being the replacement of the phosphodiester (PO) bridge by

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a phosphorothioate (PS) linkage. Unfortunately, the use of uniformly PS-modified oligonucleotides is often accompanied by certain disadvantages, such as reduced binding affinity and increased non-sequence-specific interactions with proteins such as DNA polymerase, human immunodeficiency virus type 1 (HIV-1) reverse transcriptase, RNase H, bFGF and others (Stein, 1996; Milligan et al., 1993). Therefore, it is desirable to reduce the number of PS linkages in antisense oligonucleotides to the minimum which is necessary to stabilize against nucleolytic degradation in order to reduce both the sequence-specific and the non-sequence-specific non-antisense effects.

Strategies for partial modification have been reported which include: (a) 3′-end-capping to protect against 3′-exonuclease activity which is the major and fast-cleaving nuclease in serum, (b) end-capping at both the 3′- and the 5′-end to protect against both types of exonucleases (Hoke et al., 1991) and (c) random variation of the PS content of oligonucleotides or the use of co-oligomers, such as alternating PO–PS linkages (Ghosh et al., 1993). None of the protection schemes could efficiently prevent degradation by the additional endonuclease activity present in the serum and inside cells. Consequently, neither type of oligonucleotide modified according to the three strategies was as effective in inhibiting viral proliferation in vitro as the uniformly PS-modified oligonucleotides, although the antiviral effects of the latter proved not to be sequence-dependent (Milligan et al., 1993).

We have recently reported on a new 'minimal' protection strategy for antisense oligonucleotides which is a combination of the end-capping technique and the protection of internal pyrimidine residues which are the major sites of endonuclease degradation (Peyman and Uhlmann, 1996). This strategy reduces significantly the number of PS linkages needed to make a nuclease-resistant oligonucleotide. Here, we compare properties such as nuclease stability, binding affinity (T_m) and cellular uptake of antisense oligonucleotides directed against the translation initiation site of IE110 of herpes simplex virus type 1 (HSV-1) (Peyman et al., 1995a) that are PS-modified by various protection strategies. For assessing in vitro activity, we investigated the potential of these oligonucleotides to reduce the infective yield of HSV-1 in Vero cells. We show that 'minimally' protected (end-capping and protection at pyrimidine sites) oligonucleotides are as active against HSV-1 in a de novo infection assay in vitro as uniformly PS-modified oligonucleotides, without showing the non-sequence-dependent antiviral effect usually associated with uniformly PS-modified oligonucleotides. The strong correlation between antiviral activity and nuclease resistance emphasizes the dominant role of nuclease degradation in biological experiments compared with other parameters.

Table 1 shows the positions of the PS linkages in the oligonucleotides used for this study and their antiviral efficacy in a HSV-1 cytopathic effect assay using Vero cells. The sequence used here is directed against the translation initiation codon of HSV-1 immediate early gene IE110 which proved to be highly efficient for blocking the spread of HSV-1 in vitro (Peyman et al., 1995a).

The results range from inactive (>80 μM) for the unmodified PO oligonucleotide AO # 1 to a minimum inhibitory concentration (MIC) of 1 μM for the all-PS oligonucleotide AO # 12. Replacement of three PO with PS bonds at the 3′-end (AO # 4) increases the antiviral efficacy to an MIC of 27 μM. When PS bonds are introduced at both the 3′- and the 5′-end, the antiviral activity rises with the number of PS bonds: AO # 2 (80 μM) < AO # 3 (27 μM) < AO # 5 (9 μM). When additional PS linkages are introduced in the middle of AO # 3 at pyrimidine positions, the antiviral activity rises again: AO # 3 (27 μM) < AO # 6 (9 μM) < AO # 7 (3 μM) < AOs # 8,9 (1 μM). AOs # 8 and # 9 are as active as the fully modified PS oligonucleotide AO # 12. On the other hand, there is no gain in antiviral efficacy with the additional protection of purine positions, as in AO # 10 (MIC = 27 μM) relative to AO # 3. The potency of the PO–PS co-oligomer AO # 11 is only moderate (MIC = 9 μM) when compared with the pyrimidine-protected oligonucleotide AO # 8 (MIC = 1 μM). The control oligonucleotide AO # 13 (corresponding to AO # 8 with two 'G's changed into

Table 1
Anti-HSV-1 activity of oligonucleotides of different chemical derivatization

No.	Sequence/position of PS linkage[a]	MIC[b] (μM)	n[c]
AO # 1	G C G G G G C T C C A T G G G G G T C G	>80	0
AO # 2	G*C G G G G C T C C A T G G G G G T C*G	80	2
AO # 3	G*C*G G G G C T C C A T G G G G G T*C*G	27	4
AO # 4	G C G G G G C T C C A T G G G G G G*T*C*G	27	3
AO # 5	G*C*G*G G G C T C C A T G G G G G G*T*C*G	9	6
AO # 6	G*C*G G G G C T C C*A T G G G G G G T*C*G	9	5
AO # 7	G*C*G G G G C*T C C A*T G G G G G G T*C*G	3	6
AO # 8	G*C*G G G G C*T C*C*A*T G G G G G G T*C*G	1	8
AO # 9	G*C*G G G G C*T*C*C*A*T G G G G G G T*C*G	1	9
AO # 10	G*C*G G G*G C T C C A T G*G*G*G G G T*C*G	27	8
AO # 11	G*C*G G*G G*C T*C C*A T*G G*G G*G T*C*G	9	11
AO # 12	G*C*G*G*G*G*C*T*C*C*A*T*G*G*G*G*G*T*C*G	1	19
AO # 13	G*C*G G A G C*T C*C*A*T G G A G G T*C*G	>80	8

Oligonucleotides were prepared and characterized as described in Peyman and Uhlmann, 1996; the HSV infectious yield assay was carried out as described in Peyman et al., 1995a.
[a] Sequences are written from 5′ to 3′.
[b] MIC: minimal inhibitory concentration. Maximum tolerated concentrations were in all cases >80 μM.
[c] Number of PS linkages.
* Indicates the position of a phosphorothioate linkage.

'A's), containing the same number and position of PS bonds as AO # 8, is inactive (MIC > 80 μM).

It is interesting to follow the change in properties that go with an increasing degree of modification (Table 2). Cellular uptake and binding affinity seem to depend only on the PS content of the oligonucleotide. The uptake of oligonucleotides, as measured by counting the cell-associated radioactivity, is diminished with increasing PS content; however, the difference in uptake between the unmodified AO # 1 and the uniformly modified AO # 12 is only by a factor of two. With increasing PS content, the binding affinity to a target strand (measured as T_m against the complementary DNA oligonucleotide) is also diminished. As expected, there is an average depression in T_m of -0.4°C/PS bond. In contrast, we have shown (Peyman and Uhlmann, 1996) that nuclease resistance is a function of the position rather than of the number of PS linkages contained within an oligonucleotide. It is striking that AO # 8 is a lot more resistant towards nucleolytic degradation than AO # 10 despite the fact that both oligonucleotides contain the same number of PS bonds.

A similar behaviour is found for the antiviral efficacy. On the one hand, the antiviral activity increases with the number of PS linkages introduced at the 5′- and 3′-end, as in the series AO # 1 < AO # 2 < AO # 3 < AO # 5. On the other hand, AO # 11 contains 11 PS linkages and is antivirally less efficient than AO # 8 with only eight PS linkages. In addition, AO # 8 is almost 30 times more active than AO # 10 and both oligonucleotides contain the same number of PS linkages. Both oligonucleotides must also be compared with the end-capped AO # 3 from which they are derived: the additional modifications in AO # 10 at the purine positions do not result in an increased antiviral activity. In contrast, the additional modifications in AO # 8 at the pyrimidine positions clearly improve the antiviral efficiency. This effect is verified in the series AO # 3 < AO # 6 < AO # 7 < AO # 8, which shows increasing antiviral activity with an increasing number of pyrimidine positions protected by PS replacement of the natural phosphodiester bond. However, at a certain point there seems to be a saturation: AO # 9 is not more active than AO # 8 and even the uniformly modified oligonucleotide AO # 12 shows the same activity. The

Table 2
Melting temperatures (T_m), nuclease stability and uptake levels for PO or PS modified oligonucleotides

No.	T_m [a] (°C)	$t_{1/2}$ [b] (h)	Uptake level[c] (pmol/10^5 cells)	n[d]
AO # 1	73.5	3	5.6	0
AO # 3	72.0	8	5.3	4
AO # 5	71.2	18	N.d.	6
AO # 8	71.0	30	3.2	8
AO # 10	71.1	13	N.d.	8
AO # 11	69.9	35	N.d.	11
AO # 12	65.8	>48	2.5	19

[a] Melting curves were measured against the complementary DNA oligonucleotide at 1 μM, each strand in 140 mM NaCl, 10 mM HEPES (pH 7.5) at 15–85°C, heating rate 1°C/min on a Hewlett Packard 8452 Diode Array spectrophotometer. All samples were pre-melted at 85–90°C to destroy secondary structure and then allowed to thermally equilibrate.
[b] $t_{1/2}$, Half-life in hours of nucleolytic degradation of oligonucleotides during incubation in serum ($t_{1/2}$-values are taken from Peyman and Uhlmann, 1996).
[c] Cellular uptake by Vero cells was measured as described in Peyman et al., 1995b.
[d] Number of PS linkages.

antiviral efficacy almost parallels the nuclease resistance of the examined oligonucleotides.

A particularly interesting point is that AO # 8 exhibits higher antiviral activity than the alternating co-oligomer AO # 11, although it is less nuclease-resistant. The stabilization against both exo- and endonucleolytic degradation is sufficiently high for both derivatives, while other parameters, such as cellular uptake and binding affinity, are likely to determine the observed biological activity. It seems that the higher duplex stability of AO # 8, combined with higher cellular uptake, is responsible for this effect. A further argument could be, that the duplex of AO # 8 with the target mRNA is more sensitive towards RNase H degradation than the one with AO # 11. The beneficial effect of increasing nuclease resistance by increasing the number of PS bonds is hence in part compensated by a decreased thermal duplex stability, decreased cellular uptake and, possibly, by decreased activation of RNase H. This might explain the similar antiviral efficiencies of AOs # 5, # 6 and # 11. Therefore, it is necessary to limit the number of PS linkages to especially nuclease-sensitive positions within oligonucleotides, i.e. the 5′- and 3′-end, and the pyrimidine positions.

It is important to note that the antiviral activity of AO # 8 is also highly sequence-dependent: the replacement of 'G's by 'A's as in AO # 13 (which serves as a control oligonucleotide) abolishes the antiviral activity completely. However, this does not prove an antisense-mechanism: replacement of a G-quartet by GGAG could also abolish antiviral activity caused by a non-antisense mechanism of the G-quartet (Stein, 1996), although the G-quartet effect seems to be most pronounced for uniformly PS-modified oligonucleotides. Additionally, cellular uptake of oligonucleotides is decreased if the G-quartet is destroyed (Peyman et al., 1995b).

In summary, we have shown that nuclease resistance is the dominant factor that determines the antiviral efficacy of a series of oligonucleotides with the same sequence but with different PS protection strategies. Binding affinity and cell uptake play only a minor role in this particular case. The minimal protection strategy presented earlier (Peyman and Uhlmann, 1996), consisting of end-capping and pyrimidine protection, proves to be advantageous in this HSV-1 in vitro assay as well, because it also minimizes non-sequence-dependent effects. The control oligonucleotide AO # 13, which is modified exactly as AO # 8, is inactive, while any all-PS oligonucleotides tested show activity at an MIC of 1–10 μM in our de novo infection assay (data not shown) regardless of its sequence, which is in agreement with previous observations (Milligan et al., 1993).

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