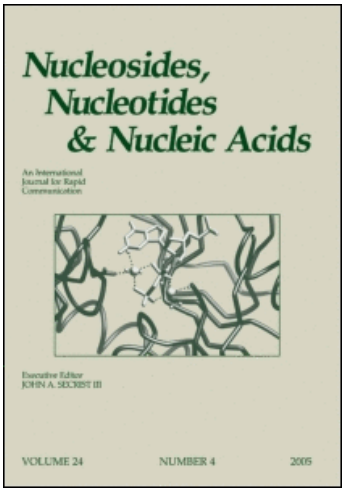


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NUCLEOSIDES & NUCLEOTIDES, 16(7-9), 1717-1720 (1997)

# ENHANCED BIOLOGICAL ACTIVITY OF ANTISENSE OLIGONUCLEOTIDES CONTAINING 5-(1-HEXYNYL)-SUBSTITUTED PYRIMIDINE NUCLEOTIDES

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**ABSTRACT.** Antisense oligonucleotides directed against HSV-1 immediate early gene mRNA were synthesized with replacement of the internal pyrimidine nucleotides by the corresponding 5-(1-hexynyl) analogues and with end-capping by phosphorothioates. These compounds were found to have improved binding affinity, increased stability towards nucleases and enhanced antiviral activity in a cell culture assay.

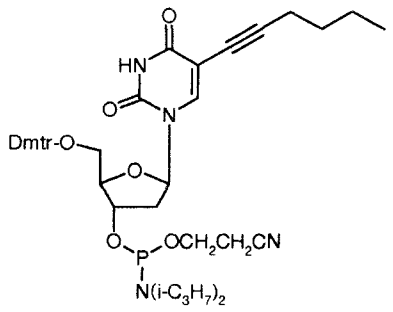


Biological activity of antisense oligonucleotides is determined by a number of parameters including binding affinity, induction of RNase H for cleavage of the target mRNA, stability against nucleases as well as cellular uptake [1]. Oligonucleotides containing C5 propynyl analogues of 2'-deoxyuridine and 2'-deoxycytidine have been shown to have enhanced binding affinity to complementary DNA and RNA [2]. In cell culture systems the C5 propynyl all-phosphorothioate oligonucleotides are more potent antisense inhibitors than the corresponding oligomers containing natural bases [3, 4]. However, in antiviral assay systems the use of uniformly phosphorothioate modified oligonucleotides is often accompanied by non-sequence-specific effects [5,6]. In order to minimize these side effects, we have recently introduced a "minimal" protection strategy for antisense oligonucleotides [7]. This strategy involves the stabilization of oligonucleotides against exonucleases by end-capping and protection at internal pyrimidine residues, which are the major sites of endonuclease degradation, by a minimal number of phosphorothioate linkages.

In this study, we have investigated oligonucleotides which are protected against exonuclease degradation by two phosphorothioate residues at both the 3'- and 5'-ends, while the internal pyrimidines were replaced by C5 alkynyl pyrimidines. Our interest was focused on oligonucleotides containing 5-(1-hexynyl)-substituted pyrimidine nucleotides whose binding affinity is only slightly lower as compared to their propynyl analogues [8], but which may have

different biological activity due to the larger substituent at C-5, potentially influencing cellular uptake and resistance towards nucleases.

Starting from 5-(1-hexynyl) thymidine and 5-(1-hexynyl) deoxycytidine we prepared the protected synthons **1-2** (R = benzoyl) which were used for the preparation of oligonucleotides ***AO2-AO7*** and control oligonucleotides ***Ctrl1*** and ***Ctrl2*** following standard synthesis protocols. The sequence used in this study is directed against the translation initiation codon of the HSV-1 immediate early gene which proved to be highly efficient for blocking the spread of HSV-1 *in vitro* [9]. All compounds were analyzed by negative ion electrospray mass spectroscopy which in all cases confirmed the calculated mass.

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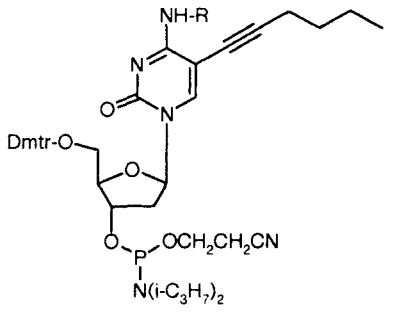


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The antiviral potency of the oligonucleotides was determined using an HSV-1 cytopathic effect assay in Vero cells. The end-capped oligonucleotide ***AO1*** showed only moderate antiviral activity (TABLE 1), whereas modification of the internal pyrimidine residues by 5-alkynyl substituents significantly improved the antiviral efficacy. Interestingly, the hexynyl modified oligonucleotides ***AO3***, ***AO5*** and ***AO7*** appeared to be more potent than the corresponding propynyl modified oligomers in this assay. The observed biological effect was sequence-dependent, as the two control oligonucleotides ***Ctrl1*** and ***Ctrl2***, in which two G's have been changed to A's, did not show any antiviral activity in our assay.

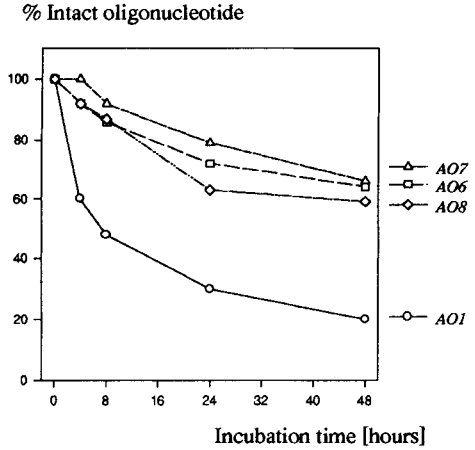
Measurement of the melting temperatures *Tm* revealed that binding affinity is higher for the propynyl derivatives than for the hexynyl derivatives. Therefore, other parameters, such as endonuclease stability, may dominate the potency of the hexynyl modified oligomers. Consequently, we have determined serum stabilities of end-capped ***AO1***, end-capped/propynyl modified ***AO6*** and end-capped/hexynyl modified ***AO7***, respectively (FIGURE 1). In fetal calf serum (FCS) we observed enhanced nuclease stability of the C5-alkynyl pyrimidine modified oligomers over oligonucleotides having only natural nucleobases. The hexynyl modified oligomer ***AO7*** turned out to be as stable as ***AO8*** which was stabilized by two phosphorothioate residues at either end and additional four phosphorothioate linkages at the

TABLE 1. Anti-HSV-1 activity and melting temperatures (*Tm*) of pyrimidine modified oligonucleotides.

| AO# | Oligonucleotide Sequence (* means phosphorothioate) | MIC [μM] | $T_m$ [°C] |
|---|---|---|---|
| *AO1* | G*C*G G G G C T C C A T G G G G G T *C*G | 25.0 | 72.0 |
| *AO2* | G*C*G G G G C $\underline{U}^{p}$C C A $\underline{U}^{p}$G G G G G $\underline{U}^{p}$*C*G | 10.0 | 76.4 |
| *AO3* | G*C*G G G G C $\underline{U}^{h}$C C A $\underline{U}^{h}$G G G G G $\underline{U}^{h}$*C*G | 5.0 | 73.1 |
| *AO4* | G*C*G G G G $\underline{C}^{p}$T $\underline{C}^{p}\underline{C}^{p}$A T G G G G G T *C*G | 10.0 | 79.8 |
| *AO5* | G*C*G G G G $\underline{C}^{h}$T $\underline{C}^{h}\underline{C}^{h}$A T G G G G G T *C*G | 2.5 | 77.3 |
| *AO6* | G*C*G G G G $\underline{C}^{p}\underline{U}^{p}\underline{C}^{p}\underline{C}^{p}$A $\underline{U}^{p}$G G G G G $\underline{U}^{p}$*C*G | 10.0 | 82.3 |
| *AO7* | G*C*G G G G $\underline{C}^{h}\underline{U}^{h}\underline{C}^{h}\underline{C}^{h}$A $\underline{U}^{h}$G G G G G $\underline{U}^{h}$*C*G | 2.5 | 79.0 |
| *AO8* | G*C*G G G G C*T C*C*A*T G G G G G T *C*G | 1.2 | 70.8 |
| *Ctrl1* | G*C*G G A G $\underline{C}^{p}\underline{U}^{p}\underline{C}^{p}\underline{C}^{p}$A $\underline{U}^{p}$G G A G G $\underline{U}^{p}$*C*G | >25 | - |
| *Ctrl2* | G*C*G G A G $\underline{C}^{h}\underline{U}^{h}\underline{C}^{h}\underline{C}^{h}$A $\underline{U}^{h}$G G A G G $\underline{U}^{h}$*C*G | >25 | - |

$\underline{U}^{p}$: 5-(1-propynyl)-dU; $\underline{U}^{h}$: 5-(1-hexynyl)-dU; $\underline{C}^{p}$: 5-(1-propynyl)-dC; $\underline{C}^{h}$: 5-(1-hexynyl)-dC. Antiviral activity was determined as reported [9]. MIC: Minimal Inhibitory Concentration. Melting curves were measured against the complementary DNA oligonucleotide at 1 μM concentration in 140 mM NaCl, 10 mM HEPES, pH 7.5 at 15-90°C.






Nuclease degradation of oligonucleotides was carried out in RPMI 1640 (Biochrom) containing 20% FCS. Oligonucleotides were added to a final concentration of 250 μg/ml. Following incubation at 37°C, aliquots were removed after 0, 4, 8, 24 and 48 hours, added to 10 μl 80% formamide solution and heat-deactivated at 95°C for 5 minutes. Electrophoresis was carried on 20% polyacrylamide / 7M urea slab gels.

FIGURE 1. Serum stability of oligonucleotides of different chemical modification.

internal pyrimidine nucleosides according to the "minimal protection" strategy. We conclude that modification of the internal pyrimidine residues by 5-hexynyl substituents can improve the stability of oligonucleotides against degradation by serum nucleases. We have shown previously the importance of nuclease stability for antiviral efficacy [10].

By replacing the natural pyrimidines in our end-capped oligonucleotides by C5 hexynyl pyrimidines we were able to enhance anti-HSV-1 activity of these compounds in a sequence-dependent manner. In contrast, any all-phosphorothioate oligonucleotide tested in the cytopathic effect assay was active in our assay regardless of its sequence (data not shown).

In summary, we have shown that replacement of the natural pyrimidines by C5-hexynyl modified pyrimidines in oligonucleotides results in enhanced binding to complementary nucleic acids. Increased resistance of hexynyl pyrimidine modified oligonucleotides towards nucleases combined with improved binding properties is likely to be responsible for the enhanced biological activity observed for these compounds. From our study, we cannot exclude that other mechanisms than antisense contribute to the detected antiviral activity. However, in recent experiments we have successfully used the hexynyl pyrimidine modified oligonucleotides for the inhibition of expression of different cellular genes, in which cases an antisense mechanism of action could be established.



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