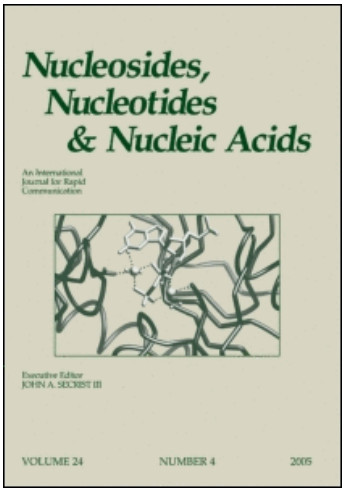


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# CONJUGATED ANTISENSE OLIGONUCLEOTIDES FOR INHIBITION OF HUMAN CYTOMEGALOVIRUS *IN VITRO*

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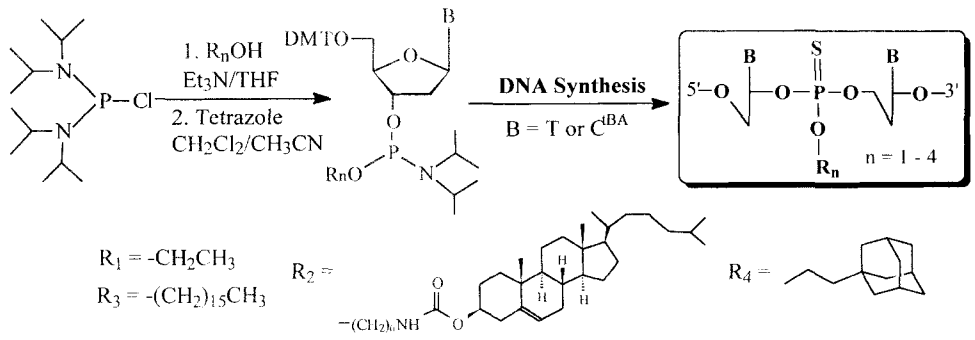
**ABSTRACT:** Several thiono triester containing oligonucleotide phosphorothioates linked with a lipophilic group have been synthesized. Some of these modified antisense oligonucleotides show potent anti-HCMV activity as well as improved cellular association and nuclease resistance.



Antisense oligonucleotides have been developed as a new class of potential antiviral agents.[1] We report herein our works on synthesis, anti-HCMV activity and pharmaceutical properties of a new class of conjugated antisense oligonucleotides. These oligonucleotides were prepared using 5'-DMT-nucleoside-*O*-alkyl-phosphoramidites, which incorporate a lipophilic group to form a non-ionic thiono triester internucleotide linkage (SCHEME 1).[2]

One advantage of using 5'-DMT-nucleoside-*O*-alkyl-phosphoramidites is the capability to incorporate thiono triester linkages and lipophilic groups in a site-specific manner. The sequence of the oligonucleotide phosphorothioates modified here is 5'-TGGGGCTTACCTTGCGAACA-3' (**UL36ANTI**), which has shown potent anti-HCMV activity.[3] Four alkyl groups (i.e., *ethyl*, *1-adamantyl-2-ethyl*, *cholesteryl-3-carboxyamino-6-hexyl* and *1-hexadecyl*) have been incorporated into oligonucleotide phosphorothioates to form a thiono triester internucleotide linkage at the 3'-end.

We have studied the anti-HCMV activity of the thiono triester modified oligonucleotides in human foreskin fibroblasts cells (HFF). The inhibition of HCMV DNA replication was analyzed by Southern blot analysis of total infected cellular DNA hybridized with a probe specific for HCMV DNA. The results are shown in TABLE 1.




**SCHEME 1.** The Synthesis of Thiono Triester Internucleotide Linkages.

**TABLE 1.** The Anti-HCMV Activity of the Modified Oligonucleotides

| Number | Sequence (3'-5') [a,b] | Activity (μM) [c] |
|---|---|---|
| **UL36ANTI** | TGGGGCTTACCTTGCGAACA | 0.1 |
| **1** | TGGGGCTTACCTTGCG**$A_1A_1C_1$**A | 0.4 |
| **2** | TGGGGCTTACCTTGCGAA**$C_2$**A | 0.1 |
| **3** | TGGGGCTTACCTTGCGAA**$C_3$**A | 0.4 |
| **4** | TGGGGCTTACCTTGCGAA**$C_4$**A | 0.1 |
| **UL364×4** | UGGGGCTTACCTTGCGAACA | 0.05 |
| **5** | UGGGGCTTACCTTGCGAA**$C_2$**A | 0.05 |
| **SM** [e] | ACCCCGAATGGAACGCUU**$T_2$**U | *d* |

[a] N indicates the 2'-O-Methyl nucleotide. [b] The subscript number indicates the triester linkage at 3' side: $R_1$= ethyl; $R_2$= Cholesteryl-3-carboxyamino-6-hexyl; $R_3$=1-hexadecyl; $R_4$=1-adamantyl-2-ethyl. [c] Concentration that gave greater than 90% inhibition of DNA replication. [d] No inhibition at highest concentration tested (0.8 μM). [e] More controlled oligonucleotides were studied.[2,3]

As the results show, the oligonucleotides (**2** and **4**) linked with a cholesteryl or adamantyl moiety at the 3' end gave greater than 90% inhibition of DNA replication at 0.1 μM, and were equally potent as the unmodified oligonucleotide **UL36ANTI**. The other modified oligonucleotides (**1** and **5**) showed less antiviral activity. Oligonucleotide **5** containing 2'-*O*-methyl nucleotides at both the 3'- and 5'-ends showed antiviral potency (0.05 μM) as high as the corresponding unmodified compound **UL364×4**.

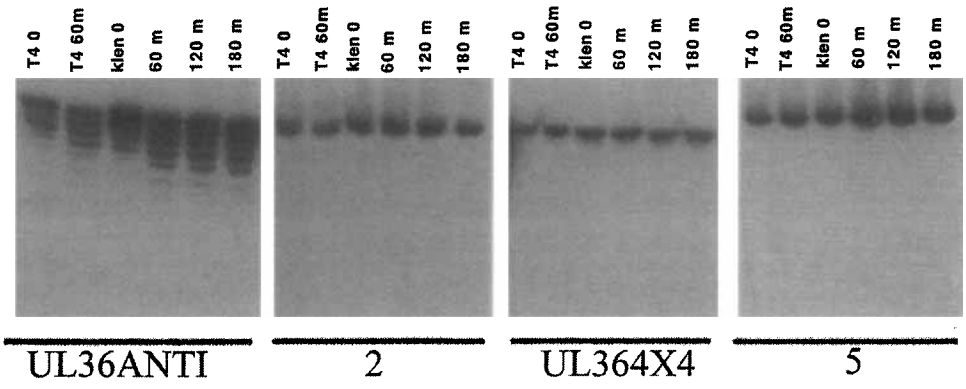



**FIG. 1**. Digestion of Oligonucleotides by Exonucleases.



The nuclease sensitivities of the oligonucleotide phosphorothioates were also studied (FIG. 1). Since degradation of oligonucleotide phosphorothioates occurs with enzymes primarily on the 3' end,[4] T4 and Klenow exonucleases were chosen for comparative digestion studies. The results clearly show that the thiono triester modified phosphorothioate **2** is much more nuclease resistant than the unmodified phosphorothioate **UL36ANTI**. The conjugated oligonucleotide **5** and the unmodified **UL364×4** containing 2'-*O*-methyl nucleotides at both the 3'- and 5'-ends also showed enhanced resistance.

The cell association, melting temperature and molecular weight of the cholesteryl conjugated oligonucleotides were studied and compared to the unmodified oligonucleotides (TABLE 2). The results show that although thiono triester linkage is neutral, introduction of a cholesteryl group into this sequence reduces the melting temperature about 2-3 °C. The cellular association of these oligonucleotides was compared by flow cytometry. HFF cells were incubated with the corresponding fluorescein labeled oligonucleotides for 24 h. The results show that HFF cellular association was significantly enhanced for cholesteryl modified oligonucleotides (**2** and **5**). The experimental data also indicated that HCMV infection had little effect on the degree of cell association.[2c] Cell uptake was studied using the fluorescein labeled oligonucleotides. Confocal microscopy studies confirmed that both modified and

**TABLE 2.** Properties of the modified CMV oligonucleotides.

| Oligomer | Cell Association[a] | $T_m$ (°C)[b] | | Molecular Ion $(M+H)^{+c}$ | |
|---|---|---|---|---|---|
| | | DNA | RNA | Calculated | Observed |
| **UL36ANTI** | 6.0 | 64.5 | 68.4 | -- | -- |
| **2** | 48.7 | 62.4 | 65.5 | 6953 | 6951 |
| **UL364×4** | 7.4 | 67.1 | 76.0 | -- | -- |
| **5** | 41.3 | 65.1 | 73.5 | 7149 | 7146 |

[a] Mean fluorescence. [b] The complementary RNA (20mer) was 5'-UGUUCGCAAGGUAAGCCCCA-3'; the complementary DNA (30mer) was added 5 bases to both the 3' and 5' ends: 5'-CCGTCTGTTCGCAAGGTAAGCCCCACGTCG-3';. [c] Analyzed by MALDI-TOF MS.



unmodified oligonucleotides were present intracellularly. It was also found that HCMV infection had changed subcellular distribution of the oligonucleotides from peri-nuclear in uninfected HFF cells to nuclear in infected HFF cells.[2c]

In conclusion, our studies demonstrate that nuclease resistance and cellular association of oligonucleotide phosphorothioates can be improved by the incorporation of a lipophilic group containing thiono triester linkage. The nature of the lipophilic group is an important factor in achieving antiviral activity. The oligonucleotide phosphorothioates modified with cholesteryl at the 3'-end exhibit potent anti-HCMV activity as well as enhanced nuclease resistance and cellular association. These properties may be fully appreciated when *in vivo* studies are completed.

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