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THIONO TRIESTER MODIFIED ANTISENSE OLIGONUCLEOTIDES FOR INHIBITION OF HUMAN CYTOMEGALOVIRUS *IN VITRO*

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Abstract. A series of thiono triester containing oligonucleotide phosphorothioates linked with a lipophilic group have been successfully 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] Human cytomegalovirus (HCMV), is widespread in the human population as a persistent subclinical infection and is becoming a major health problem due to the expansion of immunosuppressed populations.[2a] In recent years, progress has been made to develop antisense oligonucleotides for treatment of HCMV-induced retinitis in AIDS pattients.[2] We have previously demonstrated potent anti-HCMV activity of an antisense oligodeoxyribonucleotide phosphorothioate, 5'-TGGGGCTTACCTTGCGAACA-3' (**UL36ANTI**).[3] This antisense oligonucleotide is complementary to the pre-messenger RNA intron-exon donor region that encodes HCMV immediate-early proteins UL36 and UL37. We have also focused on modifying antisense oligonucleotide phosphorothioates to further improve their pharmaceutical properties.[4]

We report herein preliminary results with respect to synthesis, anti-HCMV activity, stability and cellular association of a new class of the thiono triester modified antisense oligonucleotides. A series of the thiono triester modified oligonucleotides was prepared using 5'-DMT-nucleoside-*O*-alkyl-phosphoramidites, which conjugate a lipophilic group to form a non-ionic thiono triester internucleotide linkage. It has been reported that introducing lipophilic groups at either end of the oligonucleotide may result in enhanced pharmacological properties.[5] It has been shown previously that large lipophilic groups (e.g., cholesterol and adamantane moieties) can be conjugated to the 3'-end via a short linker on the CPG, and/or attached to the 5'-end by a post-synthesis method, H-phosphonate chemistry and phosphoramidite chemistry.[5a] However, the most commonly used linkers (the corresponding phosphoramidites) do not contain nucleosides, which restricts their use as terminal modifiers. Since the nature and position of the incorporated lipophilic groups may

favorably influence the ability of the modified oligonucleotide to cross cell membranes or to hybridize effectively to target mRNA, it is desirable to introduce a non-ionic internucleotide linkage and a lipophilic group into oligonucleotides at any preselected positions (terminal/internal). One advantage of using 5'-DMT-nucleoside-*O*-alkyl-phosphoramidites, therefore, is the capability to incorporate thiono triester linkages and lipophilic groups in a site-specific manner. In this way, we can also combine the different lipophilic groups into the same oligonucleotide, which may be useful in tuning oligonucleotide structure to improve pharmaceutical properties. The sequence of the oligonucleotide phosphorothioates modified here is 5'-TGGGGCTTACCTTGCGAACA-3' (**UL36ANTI**). 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 (Figure 1).[4] All the triester containing oligonucleotides were synthesized on 1 μmol scale using an automated oligonucleotide synthesizer (Millipore 8909 Expedite™, Bedford, MA). The oligonucleotides were analyzed by gel-capillary electrophoresis and purified by PAGE. PAGE purified samples were desalted using SEP-PAK™ cartridges (Waters Corporation, Milford, MA).

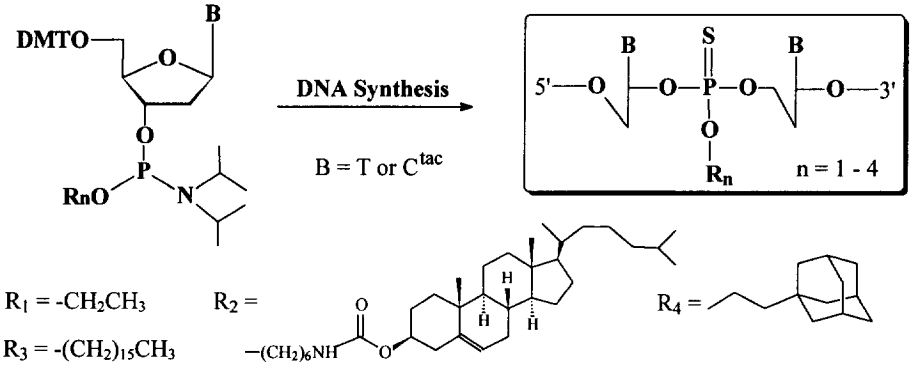

Figure 1. The Synthesis of Thiono Triester Internucleotide Linkages.

We have studied the anti-HCMV activity of the thiono triester modified oligonucleotides in human foreskin fibroblasts cells (HFF). For standard antiviral evaluation, cells were pretreated with the corresponding concentration oligonucleotides for 15 h prior to infection. Following incubation, the oligonucleotide was removed by washing 3 times with PBS and the cells were infected with HCMV (strain AD 169) using a multiplicity of infection of 0.4. 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.

Table 1. The Anti-HCMV Activity of the Modified Oligonucleotides

Number	Sequence (3'-5') [a,b]	Backbone	Antiviral Activity (μM) [c]
UL36ANTI	TGGGGCTTACCTTGCGAACA	P=S	0.1
1	TGGGGCTTACCTTGCG$\mathbf{A_1A_1C_1}$A	P=S	0.4
2	TGGGGCTTACCTTGCGAA$\mathbf{C_2}$A	P=S	0.1
3	$\mathbf{T_1}$GGGGCTTACCTTGCGAA$\mathbf{C_2}$A	P=S	0.1
4	TGGGGCTTACCTTGCGAA$\mathbf{C_3}$A	P=S	0.4
5	$\mathbf{T_1}$GGGGCTTACCTTGCGAA$\mathbf{C_3}$A	P=S	0.4
6	TGGGGCTTACCTTGCGAA$\mathbf{C_4}$A	P=S	0.1
UL364X4	UGGGGCTTACCTTGCGAACA	P=S	0.05
7	UGGGGCTTACCTTGCGAA$\mathbf{C_2}$A	P=S	0.05
8	UGGGGCTTACCTTG$\mathbf{C_2}$GAACA	P=S	0.2
9	UGGGGCTTAC$\mathbf{C_2}$TTGCGAACA	P=S	0.2
10	$\mathbf{T_2}$GGGGCTTACCTTGCGAACA	P=S	0.2
11	TGGGGCTTACCTTGCGAA$\mathbf{C_2}$A	P=O	*d*
12	$\mathbf{T_1}$GGGGCTTACCTTGCGAA$\mathbf{C_2}$A	P=O	*d*
UL36NONSENSE	ACAAGCGTTCCATTCGGGGT	P=S	*e*
NS	TCTGGGTAATTACAGCAAGC	P=S	*e*
UL36SENSE	TGTTCGCAAGGTAAGCCCCA	P=S	*e*
SM	ACCCCGAATGGAACGCUU$\mathbf{T_2}$U	P=S	*e*

[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] Concetration that gave greater than 90% inhibition of DNA replication. [d] Less than 40% inhibition of DNA replication at 0.8 μM. [e] No inhibition at highest concentration tested (0.8 μM).

The modified antisense oligonucleotides (**1-12**) in Table 1 can be divided into three groups according to their structures. The first group (**1-6**) consists of the thiono triester modified phosphorothioates which are linked to the different lipophilic groups at the 3'-end or both the 3'- and 5'-ends. To minimize changes in the geometry of the internucleotide bond and maintain the stability and fidelity of oligomer binding, only one triester linkage having a bulky group (1-adamantyl-2-ethyl, cholesteryl-3-carboxyamino-6-hexyl and 1-hexadecyl) was incorporated into the oligonucleotides. In the oligomers **3** and **5**, an additional ethyl was linked to 5'-end. As the results show, the oligonucleotides (**2**, **3**, and **6**) linked with a cholesteryl or adamantyl moiety 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**, **4**, and **5**) showed decreased antiviral activity. The second group of oligonucleotides (**7-10**) contain 2'-*O*-methyl nucleotides at both the 3'- and 5'-ends to futher improve their nuclease stability and binding affinity.[6] In these oligomers, the cholesteryl conjugated thiono triester linkage was incorporated into the internal, 3'- or 5'-end position, respectively. The most active compound in this group was oligonucleotide **7** modified at the 3'-end, which showed potency (0.05 μM) as high as the corresponding unmodified compound (**UL364X4**). The oligonucleotides modified at other positions (the 5'-end and internal) had lower activity. The third group consists of two triester modified phosphodiester oligonucleotides (**11** and **12**). We explored this type of oligonucleotide based on the increased binding affinity as well as enhanced resistance to 3'-exonuclease exhibited in comparison to the unmodified phosphorothioate.[4] As Table 1 shows, however, their anti-HCMV activities were very poor. The four control oligonucleotides (**UL36NONSENSE, NS, UL36SENSE,** and **SM**) were also studied, and had no effect on HCMV DNA replication at even the highest concentration tested (0.8 μM).

The nuclease sensitivity of the unmodified (**UL36ANTI**) and thiono triester modified phosphorothioate (**2** and **3**) were studied. Since degradation of phosphorothioates oligonucleotides occurs with enzymes primarily on the 3' end,[7] T4 and Klenow exonucleases were chosen for comparative digestion studies.[4,8] The results clearly show that the thiono triester modified phosphorothioates (**2** and **3**) are much more nuclease resistant than the unmodified phosphorothioate (**UL36ANTI**).

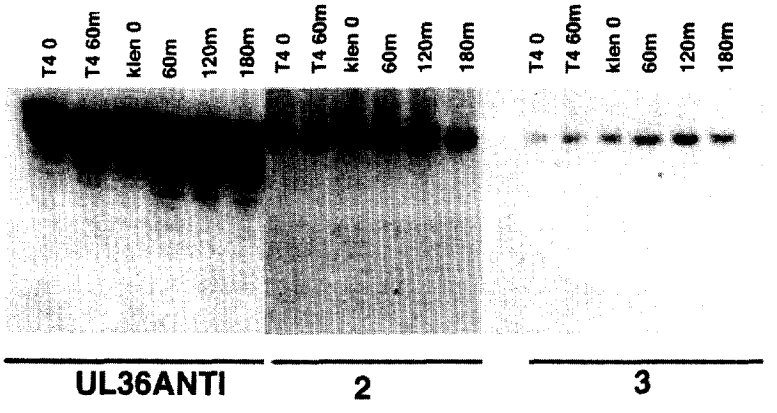

Figure 2. Digestion of Oligonucleotides by Exonucleases.

The cellular association of the unmodified oligonucleotides (**UL36ANTI** and **UL364X4**) and modified cholesteryl containing oligonucleotides (**2**, **3**, and **7**) was compared by flow cytometry.[9] HFF cells were incubated with the corresponding fluorescein labeled oligonucleotides for 4 h or 24 h. Figure 3 shows that

HFF cellular association was significantly enhanced for cholesteryl modified oligonucleotides (**2**, **3** and **7**).

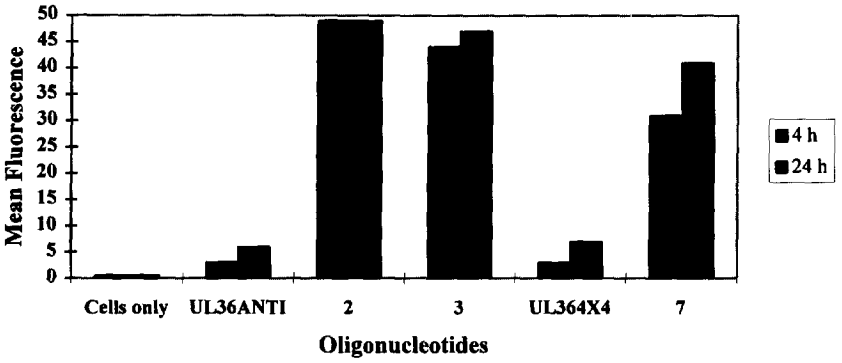

Figure 3. Cell Association of the Antisense Oligonucleotides

Since the cholesteryl conjugated oligonucleotides have a higher degree and an increased rate of cellular association, we investigated whether these properties could effect the amount of pretreatment time needed to achieve anti-HCMV activity (Figure 4). Previously we demonstrated that pretreatment time of the unmodified oligonucleotide should be at least 3 h to achieve antiviral activity with respect to inhibition of HCMV DNA replication.[3] In order to address the question of decreased pretreatment time, cells were incubated with the oligonucleotide (0.4 μm) for 15 min, 30 min, 1 h and 4 h, and then washed 3 times with PBS and infected with HCMV for 1 h. Cells were then washed, and media was then replaced with neat media. As Figure 4 shows, after only a 15 min pretreatment with the cholesteryl modified oliginucleotide **7**, HCMV DNA replication was inhibited more than 70% compared to the untreated control cells, while the unmodified oligonucleotide (**UL364X4**) had almost no effect on HCMV DNA replication. After 1 h pretreatment with **7**, HCMV DNA replication was completely inhibited, compared to the 50% inhibition achieved with the unmodified oligonucleotide. After the 4 h pretreatment, complete inhibition was achieved with both the modified oligonucleotide (**7**) and the unmodified oligonucleotide (**UL364X4**).

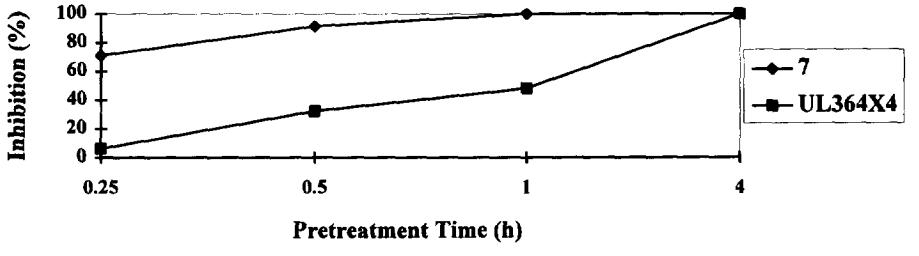

Figure 4. The Effect of the Pretreatment Time on Anti-HCMV Activity.

The results clearly show that an increase in cell association of the cholesteryl conjugated oligonucleotide correlates with a shorter pretreatment time in achieving anti-HCMV activity.

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(s). The position of incorporation and the nature of the lipophilic group are important factors 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 studies suggest that the thiono triester modified antisense oligonucleotides may exhibit superior pharmaceutical properties, however, these properties may be fully appreciated when *in vivo* studies of these oligonucleotides are done. These modified oligonucleotides are currently being studied for their binding affinity to RNA and bio-reversibility.

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8. Oligonucleotides (30 pmol) were labeled at the 5'-end with T4 polynucleotide kinase using γ-^{32}P ATP. Labeled oligonucleotides were incubated with either T4 DNA polymerase (7.5 U) or Klenow (5 U) for times indicated. Samples were analyzed by PAGE (20% polyacrylamide containing 8.3 M urea), and followed by autoradiography.
9. HFF cells were plated in 6 cm dishes in DMEM with 10% FBS at a density of 2×10^5 cells per dish. Cells were treated for 4 h or 24 h, trypsinized and analyzed. The efficiency of fluorescein labeling was determined by using a spectrofluorometer (excitation 488 nm, emission 520 nm). Flow cytometric data was acquired on 10,000 viable cell on live cells by forward scatter verses side scatter on an Epics XL Flowcytometer (Coulter, Hialeah, FL) at 488 nm with a 525 nm band pass filter.

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