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PHOSPHOROTHIOATE OLIGODEOXYRIBONUCLEOTIDES INHIBIT RIBONUCLEASE L THEREBY DISABLING A MECHANISM OF INTERFERON ACTION.

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Abstract: Phosphorothioate oligodeoxyribonucleotides were found to be inhibitors of the 2-5A-dependent RNase L. Inhibitory potency depended upon the chain length of the phosphorothioate oligonucleotide and was dependent on the phosphorothioate substitution pattern, but was not substantially base-dependent.

The interferon system is an important early defense against virus infections.[1] Treatment of vertebrate cells with the antiviral protein interferon and subsequent infection by certain viruses, such a encephalomyocarditis virus, induces the synthesis of 5′-triphosphorylated-2′,5′-oligoriboadenylates known as 2-5A.[2] Activation of the constitutive latent RNase L by such 2-5A molecules leads to degradation of virus RNA and the inhibition of virus growth; however, cellular RNAs are not immune from attack by the 2-5A-activated RNase L.[3] To target RNase L selectively to a specific RNA, we have conjugated 2-5A to antisense oligodeoxyribonucleotides.[4] This "2-5A-antisense" strategy has been applied to realize the targeted degradation of RNAs both in cell-free systems and in intact cells.[5] 2-5A-antisense[6] has effected the specific ablation of PKR mRNA and consequent loss of PKR protein and its signal transduction function,[7] the specific destruction of respiratory syncytial virus (RSV) mRNA,[8] genomic RNA and virus replication,[9] the selective degradation of bcr/abl mRNA in chronic myelogenous leukemia (CML) cells and suppression of CML cell growth,[10] and the inhibition of expression of telomerase RNA and the blockade of human malignant glioma cell growth in nude mice.[11]

For application to intact cells and animals, several generations of 2-5A-antisense chimeras have been described with varying degrees of resistance to degradation.[3,12,13] Phosphorothioates (PS) represent one of the most useful nuclease-resistant oligodeoxyribonucleotide modifications[14] and have allowed antisense oligonucleotides to advance to the clinical trial stage and to obtain FDA approval.[15] Therefore, we wished to explore 2-5A-antisense molecules in which the antisense domain internucelotide linkages were substituted with phosphorothioate. However, it is established[16] that phosphorothioates can have a variety of non specific effects such as inhibition of human DNA polymerase and RNase H. PS oligonucleotides also induce activation of complement and prolong activated partial thromboplastin time in vitro and in vivo.[17] Thus, we determined the effects of PS oligonucleotides on the activation of the key enzyme in the 2-5A-antisense strategy; namely, RNase L.

Results and Discussion.

We used oligodeoxyribonucleotides of the general formula shown in Figure 1. Phosphorothioates in replacement of phosphate internucleotide bonds are indicated in sequences by a lower case s between appropriate nucleotide residues where the substitution occurred. Oligonucleotides in which the phosphodiester bonds were totally replaced by PS are abbreviated as s-$(dN)_n$. All oligonucletides were synthesized using previously reported

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methods and were checked for purity by capillary gel electrophoresis.[4,12] For determinations of biological activity, we employed a method that utilizes cleavage of a radiolabelled oligoribonucleotide.[18] The primary cleavage product of the 20-mer [^{32}P]5′-pC$_{11}$UUC$_7$ is the 13-mer [P^{32}]5′-pC$_{11}$UUp. This assay has been described in considerable detail elsewhere.[19]

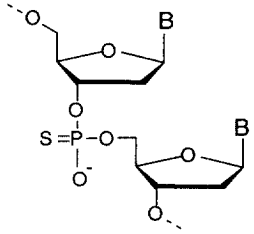

Figure 1. General formula of the oligonucleotide modifications used in this study.

In initial experiments, an all phosphodiester (PO) 25-mer oligocytidylate **1** was compared to an all phosphorothioate-substituted 25-mer oligocytidylate for their effect on the ability of the 2-5A tetramer, p5′A2′p5′A2′p5′A2′p5′A, to activate RNase L. The all PO dC$_{25}$ oligomer did not inhibit the cleavage of [^{32}P]-rC$_{11}$U$_2$C$_7$ at concentrations up to 10^{-5} M (Table 1), in keeping with the previously reported ability of 2-5A-oligodeoxyribonucleotide conjugates to activate RNase L.

In marked contrast, an all PS oligodeoxycytidylate 25-mer **7** caused a significant inhibition of the primary cleavage reaction catalyzed by RNase L. A concentration for inhibition of 50% of the substrate cleavage was defined as IC$_{50}$ and was determined in this case to be equal to 1 x 10^{-7} M from a plot of % cleavage of [^{32}P]5′-pC$_{11}$UUC$_7$ vs the concentration of added inhibitor (Table 1). This inhibition by an all PS oligonucleotide was not substantially dependent on base content. The all PS 25-mer **2** inhibited the cleavage of [^{32}P]5′-pC$_{11}$UUC$_7$ by RNase L with an IC$_{50}$ of 5 x 10^{-8} M as compared to the IC$_{50}$ of 1 x 10^{-7} M for the corresponding deoxycytidylate 25-mer (Table 1).

Next we investigated the effect of the length of the PS oligonucleotide on the potency of inhibition of RNase L. Beginning with a all PS-substituted oligocytidylate pentamer, cytidylates were added in units of five to generate a series of 8 PS oligomers up to 40-mer, including the original PS dC$_{25}$. As shown in Table 1, neither pentamer **3** or decamer **4** deoxycytidylate phosphorothioates were able to inhibit the RNase L-catalyzed cleavage of [^{32}P]5′-pC$_{11}$UUC$_7$ even when they were present at 10 μM concentration. Significant inhibition of RNase L began to be apparent when the phosphorothioate deoxycytidylate length reached 15 nucleotides (Table 1). Thus, the IC$_{50}$ of s-dC$_{15}$, **5**, was 4 x 10^{-6} M. A further increase in oligodeoxcytidylate phosphorothioate to a 20-mer resulted in increased inhibition potency to an IC$_{50}$ of 6 x 10^{-7} M (compound **6**, Table 1). As described above, the 25-mer **7** inhibited RNase L activity with an IC$_{50}$ of 1 x 10^{-7} M. Inhibitory activity plateaued with the 25-mer. Thus, the 30-mer s-dC$_{30}$ (**8**), the 35-mer s-dC$_{35}$ (**9**), and the 40-mer, s-dC$_{40}$ (**10**), were found to have IC$_{50}$'s of 2 x 10^{-7} M, 2 x 10^{-7} M and 1 x 10^{-7} M, respectively (Table 1).

In another series of experiments, the effect of various motifs of partial phosphorothioate substitution was explored. As presented in Table 1, when the 25-mer oligodeoxycytidylate was partially substituted with 8 contiguous internucleotide phosphorothioates at both the 5′- and 3′-termini (compound **11**), or when a total of 16 contiguous internucleotide linkages were PS-substituted at either the 5′- or 3′ terminus (compounds **12** and

13), potent inhibition of RNase L was still the result. The IC_{50}'s revealed that these three partially substituted congeners were slightly less inhibitory than the fully phosphorothioated oligonucleotides (Table 1). However, when the PS residues were further dispersed with intervening PO internucleotide bonds (compound **14**, Table 1), there was a further significant decrease in ability to inhibit RNase L catalytic activity.

TABLE 1
Inhibition of RNase L By Phosphorothioate Oligonucleotides

Oligo	Sequence	IC_{50}[a] Molar
1	d[CpCpCpCpC]$_5$	$> 10^{-5}$
2	d[CsTsCsTsCsGsCsAsCsCsCsAs-TsCsTsCsTsCsTsCsCsTsTsCsT]	5×10^{-8}
3	d[CsCsCsCsC]	$> 10^{-5}$
4	d[CsCsCsCsCsCsCsCsCsC]	$> 10^{-5}$
5	d[(CsCsCsCsC)$_2$CsCsCsCsC]	4×10^{-6}
6	d[(CsCsCsCsC)$_3$CsCsCsCsC]	6×10^{-7}
7	d[(CsCsCsCsC)$_4$CsCsCsCsC]	1×10^{-7}
8	d[(CsCsCsCsC)$_5$CsCsCsCsC]	2×10^{-7}
9	d[(CsCsCsCsC)$_6$CsCsCsCsC]	2×10^{-7}
10	d[(CsCsCsCsC)$_7$CsCsCsCsC]	1×10^{-7}
11	d[CsCsCsCsCsCsCsCsCpCpCpCp-CpCpCpCpCsCsCsCsCsCsCsCsC]	3×10^{-7}
12	d[CsCsCsCsCsCsCsCsCsCsCs-CsCsCsCsCpCpCpCpCpCpCpCpC]	5×10^{-7}
13	d[CpCpCpCpCpCpCpCpCsCsCsCs-CsCsCsCsCsCsCsCsCsCsCsC]	5×10^{-7}
14	d[CsCsCsCsCpCsCpCsCpCsCpCs-CpCsCpCsCpCsCpCsCsCsCsC]	1×10^{-6}

a. IC_{50} is defined as the concentration (μM) of potential inhibitor that was required to reduce cleavage of the RNA substrate [^{32}P]5′ -pC_{11}UUC_7 by 50%. Values are averages of duplicate or triplicate determinations.

The specific mechanism(s) by which inhibition by phosphorothioates operates is presently a matter of speculation. It may be possible that the PS oligonucleotides interfere with substrate RNA binding in the catalytic site. Alternatively, the phosphorothioates may interfere with the 2-5A-induced dimerization of RNase L which has been postulated to be necessary for RNase activity. It is unlikely that PS oligos interfere with the actual binding of 2-5A to RNase L as we have found (Player & Torrence, unpublished observations) that the ability of 2-5A tetramer to bind to RNase L was not affected by PS-oligonucleotides using a standard competition assay.[19]

The results of these studies lead to three important conclusions. First, oligodeoxynucleotide phosphorothioates of sufficient length (>10 - 15 nucleotides) are potent inhibitors of RNase L-catalyzed RNA cleavage. Second, the potency of inhibition shows some dependence not only on oligonucleotide phosphorothioate length, but also on extent and pattern of PS substitution. Third, these results imply that highly phosphorothioated antisense domains in 2-5A-antisense chimeras will not be compatible with effective activation of RNase L. These first three general guidelines have been exploited by Player et al.[9] in the formulation of a partially phosphorothioated 2-5A-antisense oligonucleotide potently active against respiratory syncytial virus.

Fourth, these results suggest that any highly phosphorothioate-substituted oligonucleotide that relies upon a 2-5A activation of RNase L and/or calls upon steric blocking, RNase H, aptameric, or other mechanisms to achieve biological activity, may in fact be capable of inhibiting a critical arm of the interferon mechanism by blocking the action of RNase L. Thus, such oligonucleotides, when administered as therapeutic agents, may prevent at least some of the biological activities of interferon and predispose an the recipient toward virus infections and possibly other side-effects related to a dysfunctional interferon system. This latter consideration previously has not been recognized when considering the potential toxic effects of phosphorothioate oligonucleotides.

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