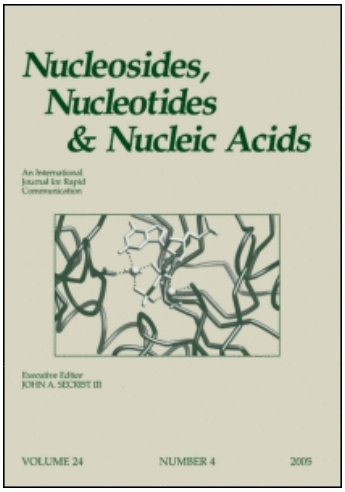

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Antisense Effects of Oligonucleotides Complementary to the Hairpin of the *Leishmania* Mini-exon RNA

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ANTISENSE EFFECTS OF OLIGONUCLEOTIDES COMPLEMENTARY TO THE HAIRPIN OF THE *LEISHMANIA* MINI-EXON RNA.

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Abstract: We investigated the binding and the translation inhibitory properties of hexadecamers complementary to the mini-exon sequence of the protozoan parasite *Leishmania amazonensis*. This targeted RNA region folds into a hairpin. Large differences were observed in the antisense properties of the different oligomers although their binding to RNA always requires the disruption of the stem region.

Leishmania are protozoan parasites responsible for severe diseases both in man and animals. The expression of their genes involves discontinuous transcription and *trans*-splicing which results in the presence of a common sequence, 39 nt long, at the 5' end of every mRNA [1]. This mini-exon (Mex) sequence is an exquisite target for antisense oligonucleotides [2] (ODN). Indeed a 16mer phosphorothioate complementary to the mini-exon sequence of *L. amazonensis* selectively killed the parasites infecting cultured murine macrophages [3,4].

The Mex sequence of *L. amazonensis* folds into a non-perfect hairpin structure, which weakens the binding of antisense ODNs [5]. It is of prime interest to design high affinity ligands which could selectively recognize folded RNA regions thus interfering with the function that these structures can ensure (see [6] for a review). In an effort to identify the most sensitive regions to antisense ODNs, we investigated the properties of a series of 16mers targeted to the Mex sequence of *L. amazonensis* (Fig. 1). All these oligomers are complementary to the stem region of the Mex hairpin. In addition, these

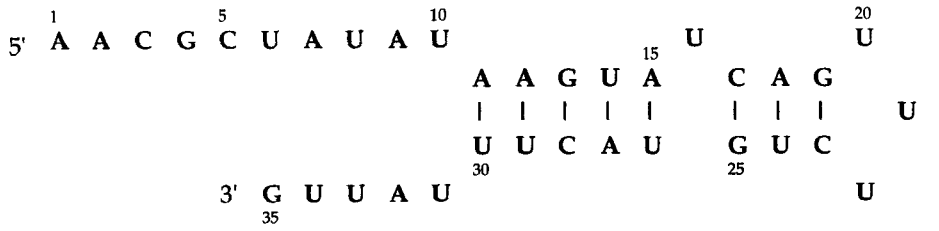

Fig 1 : Secondary structure of the mini-exon sequence of *Leishmania amazonensis*.

ODNs might engage base pairs with predicted single-stranded regions either upstream (16A, 16T5), downstream (16T3) or in the loop (16B, 16T3, 16T5) of the hairpin structure. The ODN 16B displays itself a hairpin structure which might promote loop-loop interactions with the target RNA.

The affinity of the anti-Mex ODNs was investigated by electrophoretic mobility shift assay on non-denaturing polyacrylamide gels. ^{32}P 5' end-labelled Mex RNA was incubated with the various ODNs for 30 minutes at 4°C, in a 50 mM Tris-acetate buffer (pH 7.5) containing 10 mM magnesium acetate. The formation of an ODN-Mex RNA complex resulted in a slowly migrating species. The dissociation constant was determined as the ODN concentration which shifted 50% of the labelled RNA. 16A which was used in previous studies [3, 4] was by far the strongest binder with a Kd one order of magnitude lower than that of 16T5 eventhough both ODNs have 7 bases complementary to single-stranded parts of the target RNA (Fig. 1). However for 16A the complementary bases are in a row, upstream of the Mex hairpin whereas 16T5 binds to single-stranded portions upstream (2 nt) and in the loop (5 nt). This might make a difference in the nucleation step of hybrid formation. Alternatively the hairpin loop might have some structured character whose disruption might require energy. 16B is a poor binder (Fig. 1) ; this is likely related to the self-structure of this antisense sequence. Even if this ODN can give rise to loop-loop interactions its structure likely prevents the invasion of the target stem, making 16B 100-fold less efficient that 16A (Fig. 1).

We then monitored the effect of these oligomers on *in vitro* translation of *L. amazonensis* mRNA (prepared from cultured promastigotes as previously described [7]) in wheat germ extract. The inhibitory efficiency decreased in the order 16A>16T5>16B>16T3 (Fig. 2), *i.e.* was correlated to the affinity of the ODN. The

Table 1 : Characteristics of antisense oligonucleotides used in this study. The location of the target on the mini-exon sequence, the dissociation constant (Kd) evaluated by electrophoretic mobility shift assay and the oligonucleotide concentration (C 1/2) which induces 50 % reduction of protein synthesis in a cell-free translation assay are indicated.

ODN	target	Kd (µM)	C1/2 (µM)
16 A	4-19	0.7	0.2
16 B	13-28	100	1
16 T3	18-33	> 100	2
16 T5	9-24	6	0.2

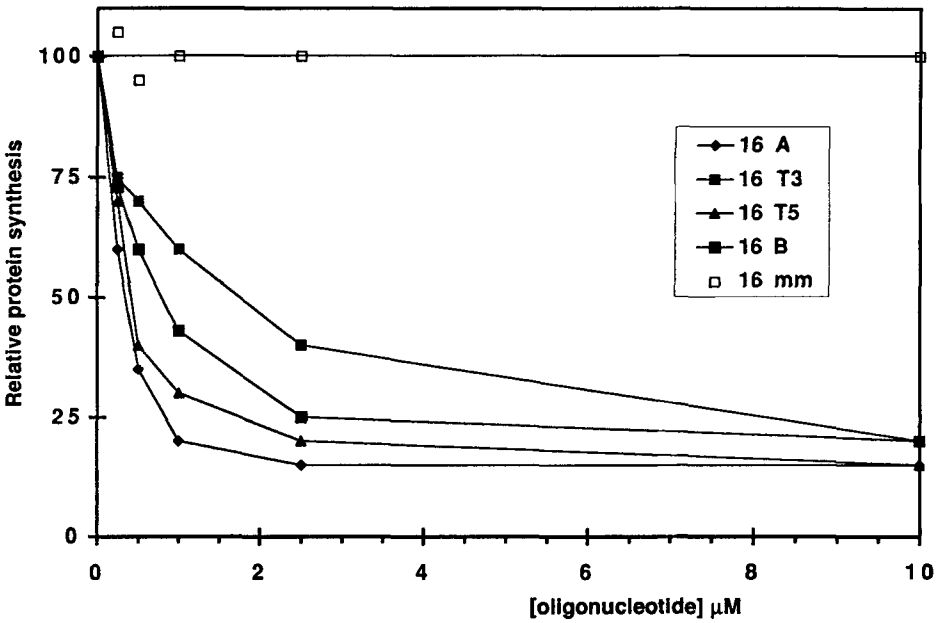

Fig 2 : Effect of anti-mini-exon oligodeoxynucleotides on *in vitro* translation of *L. amazonensis* mRNA in wheat germ extract. Unmodified oligodeoxynucleotides were obtained from Eurogentec and purified as described previously [7]. The protein was quantitated by trichloroacetic acid precipitation of ^{35}S methionine labelled proteins [7]. The mismatched oligomer 16 mm (5'CTGTTCTAATAATAGC) was used as a control.

translation inhibition was specific as i) 16mm which displays 8 mismatched bases compared to 16A had no effect on *L. amazonensis in vitro* protein synthesis (Fig. 2), ii) the anti-Mex 16mers had a weak (<10%) effect on *in vitro* translation of Brome Mosaic Virus mRNA at concentrations below 10 mM (not shown).

We have demonstrated that the Mex RNA structure has an inhibitory effect on antisense ODN in fair agreement with previous studies on various mRNAs [5, 8]. Moreover, the antisense ODN hairpin susceptible to generate a loop-loop complex did not efficiently bind to the RNA structure. A similar result was reported for the TAR RNA of HIV-1 [9]. The above results underline the difficulty to rationally optimize the target sequence in a structured RNA region. Chemically-modified oligonucleotides able to invade RNA structures [10] or strategies that take into account folded RNA such as triplex formation [11, 12] or combinatorial approaches [13-15] constitute interesting alternatives.

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