Antisense Oligonucleotides Containing Modified Bases Inhibit
in Vitro Translation of Leishmania amazonensis mRNAs by
Invading the Mini-exon Hairpin

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Complementary oligodeoxynucleotides (ODNs) that contain 2-aminoadenine and 2-thiothymines interact weakly with each other but form stable hybrids with unmodified complements. These selectively binding complementary (SBC) agents can invade duplex DNA and hydridize to each strand (Kutavin, I. V., Rhinehart, R. L., Lukhtanov, E. A., Gorn, V. V., Meyer, R. B., and Gamper, H. B. (1996) Biochemistry 35, 11170–11176). Antisense ODNs with similar properties should be less encumbered by RNA secondary structure. Here we show that SBC ODNs strand invade a hairpin in the mini-exon RNA of Leishmania amazonensis and that the resulting heteroduplexes are substrates for Escherichia coli RNase H. SBC ODNs either with phosphodiester or phosphorothioate backbones form more stable hybrids with RNA than normal base (NB) ODNs. Optimal binding was observed when the entire hairpin sequence was targeted. Translation of L. amazonensis mRNA in a cell-free extract was more efficiently inhibited by SBC ODNs complementary to the mini-exon hairpin than by the corresponding NB ODNs. Nonspecific protein binding in the cell-free extract by phosphorothioate SBC ODNs rendered them ineffective as antisense agents in vitro. SBC phosphorothioate ODNs displayed a modest but significant improvement of leishmanicidal properties compared with NB phosphorothioate ODNs.

Trypanosomatids are attractive targets for the antisense approach, as every mature transcript contains a common species-specific mini-exon sequence spliced onto its 5′ end (1, 2). Maturation of mRNAs includes a trans-splicing event, which transfers a 39-nucleotide segment of the mini-exon derived pre-RNA (the medRNA)1 to the 5′ end of every message (3).

Hybridization of an antisense oligonucleotide (ODN) to this sequence can potentially inhibit translation of all transcripts. Indeed, it was demonstrated that anti-mini-exon oligonucleotides were able to prevent translation of Trypanosoma and Leishmania mRNAs in cell-free extracts (4–6), to kill procyclic forms of T. brucei (7), and to cure L. amazonensis-infected macrophages in vitro (Refs. 8 and 9; see Ref. 2 for a review).

The trypanosomatid medRNA was proposed to adopt a secondary structure based upon conservation of folding pattern for different RNA (10). Recently the Leptomonas collosoma medRNA was shown to switch between two alternate structures (11, 12). One form leads to a base pairing pattern conserved for all of the trypanosome medRNA, suggesting critical functional interactions for splicing (11). In L. amazonensis the mini-exon was shown to fold into a structure that interferes with the hybridization of antisense ODNs (13, 14). RNA intramolecular structures that prevent the formation of oligonucleotide-RNA intermolecular complexes weaken antisense effects. This limitation has prompted the design of oligonucleotides able to overcome the mini-exon structure (for a review, see Ref. 15).

In a recent report, the L. amazonensis mini-exon sequence was efficiently complexed by an ODN capable of forming a secondary structure to form a triple strand with the putative hairpin element (16). This “double hairpin” complex readily formed at pH 6.0 using a pyrimidine motif for triplexing. However, this oligomer did not show selective inhibitory properties in cell-free translation experiments, probably due to pH conditions that are not appropriate for the formation of triplex (10). Alternatively, the disruption of the secondary structure by oligomers of high affinity can be considered.

We have been investigating selectively binding complementary (SBC) ODNs as an alternative for targeting structured nucleic acids (17, 18). These ODNs are intended to be used as complementary pairs or as a single self-complementary agent. Due to the presence of modified bases, they are unable to form stable hybrids with one another but should hybridize to normal DNA or RNA complements. We have shown that SBC ODNs containing 2-aminoadenine and 2-thiothymines can strand invade the end of double-stranded DNA in a process that is favored both kinetically and thermodynamically (17). The A/U-rich hairpin proposed for the L. amazonensis mini-exon sequence presents an ideal target for testing whether SBC ODNs can strand invade an RNA stem loop by hybridizing to every base of the element. In this study we experimentally confirmed the hairpin structure of the L. amazonensis mini-exon sequence and demonstrated that antisense ODNs with SBC character are more

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1 The abbreviations used are: medRNA, mini-exon derived pre-RNA; ODN, oligodeoxynucleotide; SBC, selectively binding complementary; NB, normal base; MPE, methylpropidium-EDTA; PO, phosphodiester; PS, phosphorothioate.
effective than normal base (NB) ODNs in addressing this hairpin. Our results show that SBC ODNs form very stable hybrids with the entire hairpin sequence, and that the heteroduplexes are substrates for Escherichia coli RNase H. We demonstrated that SBC ODN-RNA hybrids inhibit translation of L. amazonensis mRNA in a cell-free extract when RNase H is present. The successful targeting of a simple hairpin by SBC ODNs suggests that other secondary structure features in RNA should also be accessible to these ODNs.

EXPERIMENTAL PROCEDURES

Oligonucleotide Synthesis—Preparation of protected phosphoramidite precursors of 2-thiothymidine and 2-aminoadenosine and synthesis of SBC ODNs using these reagents have been described (17). NB ODNs with DNA, RNA, or 2'-O-methyl backbones were synthesized by routine procedures using commercially available chemicals. The Beaucage reagent was used to prepare NB and SBC ODNs with a phosphorothiate backbone (19). All antisense ODNs contained a 5'-hexanol end group as a consequence of using a modified hexanol primer controlled pore glass support (20). Capillary gel electrophoresis indicated that the SBC ODNs were at least 85% pure, and hydrolysates of SBC ODNs prepared as described previously (17) gave the expected ratios of nucleosides. Extinction coefficients of ODNs were calculated using a nearest neighbor model (21) and employed values of 9.800 m⁻¹ cm⁻¹ for 2-thiomydine (22) and 6.800 m⁻¹ cm⁻¹ for 2-aminoadenosine (23) at 260 nm.

RNA Synthesis—The mini-exon RNA of L. amazonensis was prepared by in vitro transcription of pBluescriptIKS in which the mini-exon sequence was cloned downstream of the T7 promoter, using Ampliscribe T7 transcription kit (Tebu). In the resulting transcript, the original mini-exon sequence was flanked by sequences derived from the pUC vector, both on the 5' and the 3' ends (5'-GAUC). The RNA was purified by electrophoresis on a 12% polyacrylamide denaturing gel.

Nuclease Mapping of Mini-exon RNA Secondary Structure—5'-End-labeled mini-exon RNA (100 pmol) was incubated for 20 min at 37 °C in the presence of 1.9 pmol of S1 nuclease (Boehringer Mannheim) in 50 mm sodium acetate buffer (pH 4.5) containing 28 mm NaCl and 4.5 mm ZnSO₄. RNases T1 and V1 (Boehringer Mannheim) digestions were performed for 10 and 20 min, respectively, in 20 mm HEPES buffer (pH 7.4) containing 140 mm KCl, 20 mm sodium acetate, and 3 mm MgCl₂ (S buffer). Cleavage by 15 mm methyl propidium-EDTA-Fe(II) complexes was carried out for 10 min at 37 °C in 5 mm HEPES buffer. RNase T1 digestion under denaturing conditions was achieved for 10 min at 50 °C in 30 mm sodium acetate and 5 mm urea. Digestion products were analyzed by electrophoresis on a 12% polyacrylamide denaturing gel followed by autoradiography.

Determination of Hybrid Melting Temperatures—Complementary ODNs were diluted in 20 mm HEPES, pH 7.2; 10 mm MgCl₂, and 140 mm KCl to give 2 μM of each ODN. Hybridization was assayed by rapid heating of the samples. The denaturation temperature in a Lambda 2 (Perkin-Elmer) spectrophotometer equipped with a PFT-6 automatic multichannel temperature programmer. Samples were heated at the rate of 0.5 °C/min. Melting temperatures (Tm) values were determined from the derivative maxima.

Determination of Hybrid Equilibrium Dissociation Constants by Electrophoretic Mobility Shift Assay—The mini-exon RNA prepared by in vitro transcription as described above was labeled by incorporating [α-32P]ATP (37.5 MBq/mmol). RNA and oligonucleotide were heated separately for 5 min at 65 °C and cooled down on ice. One pmol of RNA was mixed with the desired ODN in 15 μl of 50 mm Tris acetate buffer (pH 7.0) containing 10 mm magnesium acetate. The mixture was incubated for 15 min at 4 °C. The samples were then run in the same Tris buffer at 10 V/cm for about 15 h, on a 15% nondenaturing polyacrylamide gel at 4 °C. The activity in the bands corresponding to the free RNA oligonucleotide complexes. Prior to incubation with RNase H, the oligonucleotide and the RNA were treated as described for electrophoretic mobility shift assay. 5'-End-labeled RNA was then mixed with the desired ODN at a final concentration of 2 and 50 μM, respectively, in a 20 mm HEPES buffer, pH 7.0 containing 50 mm NaCl, 10 mm MgCl₂, and 1 mm dithiothreitol. The mixture was kept at 4 °C for 15 min prior to incubation with 0–4 units of E. coli RNase H (Promega) for 15 min at 4 °C. The reaction was stopped by adding one volume of 8 M urea. Samples were analyzed by electrophoresis on a 20% polyacrylamide gel containing 7 M urea.

In Vitro Treatment of Leishmania-infected Macrophages—Preparation of macrophages and parasites was carried out as described (8). Leishmania amazonensis (LV79 strain), prepared from infected BALB/c mice, was used to infect adherent macrophages at a multiplicity of 5 parasites/cell, resulting in more than 70% infected cells. This was normalized to 100% for comparison between different experiments. Infected macrophages were incubated at 34 °C with the desired oligonucleotide concentration for 48 h in RPMI/HEPES medium containing 10% fetal bovine serum. The cultures were then washed, fixed in methanol and acetic acid, and stained with Giemsa. Cells were observed by microscopy to determine the level of infection. About 500 macrophages were scored for each oligonucleotide concentration; a cell was identified as infected when it contained at least one recognizable parasite.

RESULTS

The Mini-exon Sequence of L. amazonensis Forms a Hairpin—The mini-exon region is known to fold into secondary structures (11, 12). We investigated the structure of a 35-nucleotide-long mini-exon sequence in which the four modified nucleotides (24) at the 5' end of the natural sequence were omitted (Figs. 1 and 2). Indirect evidence favors the existence of a stable structure in the mini-exon sequence of L. amazonensis. Anomalous electrophoretic mobility of this oligomer as well as low binding efficiency of complementary oligonucleotides have been observed previously (6, 13). Prior to initiating the targeting of this structure with NB and SBC ODNs, we confirmed its existence by footprinting. The cleavage pattern obtained with nuclease S1, RNases T1 and V1, and methylpropidium-EDTA (MPE) led to the secondary structure shown in Fig. 1. Positions 23–26, which are cleaved by S1 nuclease (lane 4), correspond to the apical loop of the imperfect hairpin. Whereas G23 was a cleavage site for RNase T1, no band corresponding to G17 and G29 was observed suggesting a structured region. This was further confirmed by RNase V1 and MPE, which preferentially cleave double-stranded structures. The reduced cleavage observed with RNase V1 compared with MPE in the upper part of the stem might be related to the bulged U20. The double-stranded stem likely extends to the U35-U37/A13-A11 region as indicated both by RNase V1 and MPE sensitivity, whereas both G8 and G39,G40 have a clear single-strandedness character. The structure of the upper part of the stem is in fair agreement with previous models (11); the differences at the bottom of the stem region are related to the different sequences used.

Model System for Evaluating SBC Antisense ODNs—The mini-exon sequence of L. amazonensis is an attractive target for evaluating SBC ODNs as antisense agents. The high A/U content (77%) of the hairpin and its flanking arms should favor the use of SBC ODNs that contain 2-aminoadenine and 2-thiothymine bases in place of adenine and thymine. These base analogs cannot hydrogen-bond to one another, due to steric clash, but can form good pairs with complementary unmodified bases (17). As a consequence, an SBC ODN complementary to the hairpin element of the mini-exon should be single-stranded and yet capable of strand invading the stem-loop structure. In

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bases complementary to the 5' arm at the base of the hairpin. SBC ODN pairs were also compared with NB ODN pairs as agents to target the mini-exon hairpin. These 15-mer ODNs were complementary to the 5' or 3' half of the hairpin (15Le-I or 15Le-II; see Fig. 2). Annealing was again promoted by making a 5-base-long segment of each ODN complementary to one of the overhangs at the base of the hairpin. Hybridization of both ODNs to the hairpin generated a 30-base pair DNA-RNA hybrid with a nick separating the ODNs. By employing two paired ODNs instead of a single self-complementary ODN, the likelihood of mutual interaction between the NB ODNs was significantly reduced and the potential advantage of the SBC pair accordingly diminished.

NB and SBC versions of 25Le or 15Le-I + 15Le-II were synthesized with phosphodiester (PO) or phosphorothioate (PS) backbones. Properties of these ODNs were compared relative to 16Le, a NB 16-mer (Fig. 2) used in a previous study (8).

**Thermostability of Hybrids Formed by NB and SBC ODNs**—Since the entire sequence of a stable hairpin was being targeted, the various antisense ODNs were prone to hairpin formation as well. Self-association of these ODNs was examined by UV-absorption thermal denaturation. While none of the SBC ODNs gave a melting transition, PO and PS versions of NB 25Le gave melting temperatures ($T_m$) of 46 and 31 °C, respectively (Table I). The NB combination of 15Le-I + 15Le-II formed a very weak hybrid when both ODNs had a PO backbone ($T_m = 13$ °C) and no hybrid at all when they possessed a PS backbone. The results obtained with antisense sequences are in fair agreement with the expected pairing properties of the ODNs; NB-containing oligomers give rise to more stable antisense structures than SBC ODNs and are therefore less likely to hybridize with the target sense sequence.

Absorption thermal denaturation was also used to compare the relative stabilities of hybrids formed by 15Le-I or 15Le-II with complementary DNA or RNA oligomers. Our results show that SBC ODNs with PO or PS backbones form more stable hybrids with DNA and RNA than the analogous NB ODNs (Table I). While the enhanced stability of SBC ODN-DNA hybrids ($\Delta T_m = 11–14$ °C) is probably attributable to the additional hydrogen bond present in 2-aminoadenine/thymine base pairs, the even greater stability of SBC ODN-RNA hybrids ($\Delta T_m = 21–28$ °C) requires further explanation. One possibility is that SBC ODNs favor the formation of an A-motif duplex with RNA. Indeed, the SBC/PO 15Le-II hybrid with RNA is similar in stability to the hybrid formed using an ODN with a 2'-O-methyl backbone, a combination that favors an A-type duplex (Table I).

**Binding of NB and SBC ODNs to the Mini-exon Hairpin**—Electrophoretic mobility shift analysis was used to compare hybridization of NB and SBC ODNs to the RNA mini-exon hairpin. Fig. 3 shows representative autoradiographs from which $K_d$ values were extracted (Table II). NB 15Le-I and NB 15Le-II were poor binding agents, with the PS analogs worse than the PO analogs. Dissociation constants for 15Le-II were in the high micromolar range: 20 and 150 μM for NB/PO and NB/PS analogs, respectively. SBC versions of the same ODNs were much more effective binding agents. For example, the $K_d$ values of hybrids formed by PO or PS versions of SBC 15Le-II were about 50- or 200-fold smaller than the $K_d$ values of the corresponding complexes formed by NB 15Le-II ODNs. This agrees fairly well with the $T_m$ values obtained with complementary RNA (see above); SBC ODNs were better able to open the hairpin structure of the mini-exon. For comparison we evaluated the binding of a standard 16-mer used in a previous study (8); the PO and PS 16Le were characterized by $K_d$ of 0.7 and 5 μM, respectively.

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**Fig. 1. Secondary structure of L. amazonensis mini-exon RNA.**

A, mapping of mini-exon RNA incubated with: RNase T1 under denaturing (lane 1) or non-denaturing conditions (lane 2), nuclease S1 (lane 4), RNase V1 (lane 5), or MPE (lane 6). The alkaline digestion of the mini-exon RNA is in lane 4. The G residues are identified to the left. B, the mini-exon hairpin structure deduced from nuclease mapping. The italicized bases correspond to regions of the plasmid used to generate the mini-exon by in vitro transcription. The mini-exon sequence is numbered with respect to the natural sequence, even though the first four modified nucleotides have been omitted in this construct.

In this case the upper 8-base pair stem of the hairpin would be replaced by 20 base pairs formed between the ODN and the RNA. Moreover, 9 of these new base pairs would be highly stable 2-aminoadenine-uracil doublets with three hydrogen bonds (25). To promote annealing, both NB and SBC ODNs were synthesized as 25-mers (25Le; see Fig. 2) with the 5 extra
The partially self-complementary 25Le ODNs provided an 15-mer to pair with each other, in comparison to NB ODNs. The SBC oligomers for RNA and to the reduced ability of SBC higher. This is probably related both to the increased affinity of a PO or PS backbone. The 3 contained uracil in place of thymine. 16Le has been previously used to target the mini-exon sequence (8) and contained a NB sequence and either bases (complements to 15Le-I and 15Le-II. 15Le-I, 15Le-II, and 25Le were synthesized with PO and PS backbones and contained adenine and thymine is at the top mini-exon RNA and the SBC versions of NB/PO SBC/PO NB/PS SBC/PS NB/2'-OMe Stabilization of hybrids formed by antisense Le ODNs with each other and with complementary DNA or RNA targets.

| ODNs and hybrids | NB/PO | SBC/PO | NB/PS | SBC/PS | NB/2'-OMe |
|------------------|-------|--------|-------|--------|-----------|
| 15Le-I + 15 Le-II | 13    | —      | —     | —      | ND        |
| 25Le             | 46    | ND     | 46    | ND     | 46        |
| 15Le-I + DNA-I   | 41    | ND     | 35    | 49     | 46        |
| 15Le-I + RNA-I   | 41    | ND     | 29    | 57     | 63        |
| 15Le-II + DNA-II | 48    | 59     | 40    | ND     | 50        |
| 15Le-II + RNA-II | 37    | 58     | 28    | ND     | 62        |

* Denotes bases and backbone composition of antisense ODNs. ND, not determined; —, no transition was detected.

**TABLE II**

Equilibrium dissociation constants for the binding of NB and SBC antisense ODNs to the mini-exon RNA of *L. amazonensis*.

| ODNs | NB/PO | SBC/PO | NB/PS | SBC/PS |
|------|-------|--------|-------|--------|
| 15Le-I | >>1  | 0.3    | >>2  | 0.2    |
| 15Le-II | 20   | 0.4    | 150   | 0.8    |
| 15Le-I + 15Le-II | 4  | 0.5    | 250   | 0.6    |
| 25Le   | 20   | 0.25   | 50    | 0.7    |

Effects of NB and SBC Antisense ODNs on in Vitro Translation in Cell-free Extracts—The NB and SBC ODNs with a PO backbone were tested for antisense activity against total mRNA from *L. amazonensis* in a cell-free translation system catalyzed by wheat germ extract. In this model system, SBC 25Le was the most effective antisense agent (Fig. 4). It elicited half-maximal inhibition at 0.08 μM (the C1/2 value). These parameters compare favorably to those of reference ODN 16Le (which has a PO backbone and contains NB bases), which has a C1/2 of about 1 μM. In repeated tests, NB 25Le failed to significantly inhibit translation (Fig. 4D). The dramatic difference in functionality between the SBC and NB versions of 25Le reflects the physical binding properties of these two ODNs (Table II). The paired 15Le-I + 15Le-II ODNs were also effective antisense agents, and here again the SBC pair was more potent than the NB pair. C1/2 of 0.5 μM was determined for the SBC ODN pair. When each 15-mer was tested alone for antisense activity, potency was reduced and no advantage of SBC over NB ODNs was detected. None of the anti-mini-exon ODNs interfered with translation of brome mosaic virus RNA (<5% inhibition at 1 μM ODN). Phosphorothioate NB and SBC antisense ODNs did not inhibit translation at concentrations below 0.5 μM (data not shown). Adsorption of these ODNs by proteins in the extract may explain why they failed to elicit an antisense effect. Following incubation with wheat germ extract, the PO SBC 25Le ODN ran identically to an untreated control on a nondenaturing polyacrylamide gel while the PS ODN ran as a slow moving smear attributable to the association with proteins from the.
extract (data not shown). Such binding has been reported by others for NB ODNs with a PS backbone (26). Moreover, upon binding to RNA, these PS sequences formed poorer substrates for RNase H than PO counterparts (see below).

**SBC-RNA Duplexes Are Substrates for RNase H**—Two different mechanisms account for the inhibition of translation by antisense ODNs: RNase H-independent (translation arrest) and RNase H-dependent cleavage of target RNA (27, 28). To investigate the mechanism by which SBC ODNs inhibit *L. amazonensis* on RNA translation, we carried out translation in

**Fig. 4. Effect of NB and SBC antisense ODNs on *in vitro* translation of *L. amazonensis* mRNA catalyzed by wheat germ extract (A–E) or rabbit reticulocyte lysate (F).** NB (●) and SBC (□) versions of the following phosphodiester ODNs were compared for antisense activity: panel A, 15Le-I; panel B, 15Le-II; panel C, 15Le-I + 15Le-II; panel D, 25Le. Panel E shows the antisense activity of the reference ODN 16Le (NB/PO). Panel F shows the activity of 25Le (SBC/PO) when using rabbit reticulocyte lysate in the absence (●) or presence (□) of *E. coli* RNase H.
rabbit reticulocyte lysate, which has a low (if any) class I RNase H activity under translation conditions (29). In Fig. 4F the effect of SBC 25Le (with a PO backbone) on translation was monitored both in the presence and absence of added E. coli RNase H. The results show that inhibition of protein synthesis in this medium occurs via an RNase H-dependent pathway.

To confirm that SBC ODNs form substrates for RNase H upon binding to a complementary RNA, we incubated end-labeled mini-exon RNA with all four versions of 15Le-I, 15Le-II, and 25Le in the presence of E. coli RNase H. The results obtained with 15Le-I and 25Le are presented in Fig. 5. ODN versions sensitized the mini-exon to hydrolysis to various extents. The amplitude of cleavage roughly paralleled the affinity of ODNs for the mini-exon sequence as determined by electrophoretic mobility shift assay, suggesting that the RNase H activity was first driven by the ability of oligonucleotides to invade the mini-exon hairpin. The NB-PS and NB-PO analogs of 15Le-I, which are very poor binders as shown both by gel electrophoresis and Tm measurements, did not induce significant cleavage of the mini-exon RNA by E. coli RNase H. In contrast, the most effective cleavage was obtained when using SBC/PO 25Le, which was also the strongest binder and a good translation inhibitor. However, PS oligonucleotides formed poorer substrates than PO ones. This is unlikely to be due to the presence of SBC bases, as SBC/PO oligonucleotides are excellent elicitors of RNase H activity.

Interestingly, different patterns of RNase H cleavage were obtained, depending on the chemical nature of the bases of the antisense oligos; similar profiles were obtained for NB oligomers on the one hand and SBC oligomers on the other hand, independent of the backbone. For instance, whereas NB/PO 25Le generated cleavage at A22, the SBC ODNs did not induce cleavage at this position. In contrast, strong sites corresponding to U24 and U25 were observed with SBC 25-mers. Other differences were also seen at the 3’ end of the RNA (compare cleavage at U28 and G29). Significant differences were also seen with the ODN 15Le-I; the digestion profile obtained with either the SBC/PO or the SBC/PS derivatives differed markedly from the one with the NB/PO version.

In Vitro Effects of SBC Anti-mini-exon Oligonucleotides on Cultured Parasites—As the presence of nucleases in the growth medium prevented us from using PO oligomers, investigations on cultured parasites were restricted to phosphorothioate derivatives. The addition of phosphorothioate anti-mini-exon oligonucleotides to the culture, either with NB or SBC character, led to the typical morphological changes previously described for 16Le (8). Oligonucleotide-treated cultures contained numerous macrophages with fragmented vacuoles devoid of any parasite (data not shown). At any tested concentration (5, 10, or 30 μM), the two NB ODNs 16Le and 15Le-I behaved similarly, curing from 10 to 30% macrophages (Fig. 6). The use of an SBC analog (15Le-I) showed some limited increased efficiency over the NB ODN. Therefore, the effect observed on cultured cells did not reflect the binding properties of the SBC and NB 15 mers (Table II). However, the full advantage of SBC sequences was expected only when both sides of the structure are targeted. Indeed, whereas the combination NB 15Le-I + NB 15Le-II did not improve the leishmanicidal efficacy, compared with the effect of a single sequence, the simultaneous addition of the two SBC 15-mers resulted in an improved leishmanicidal effect better seen at low concentration; 18 and 32% of cured macrophages were observed after incubation of infected cells with 5 μM mixture of the NB and SBC 15-mers, respectively (Fig. 6). This effect was selective as NB and SBC control phosphorothioate oligonucleotides induced a much lower effect (about 5% at 5 μM) in agreement with our previous study (8).

DISCUSSION

We have compared the binding and antisense activities of single and paired ODNs with NB and SBC character. Not surprisingly, as reported previously, antisense potency seemed to parallel hybrid stability (13, 30). SBC ODNs were superior to NB ODNs, and ODN pairs were more efficient than single ODNs. A key attribute of antisense SBC pairs is their enhanced ability to invade double-stranded nucleic acids due to their reduced propensity to hybridize with each other. We had previously postulated that the inhibitory influence of RNA secondary structure on the binding of antisense ODNs might be overcome through the use of perfectly paired SBC complements (17, 18). In this respect, the targeting of an RNA hairpin, like that...
formed by the mini-exon sequence of *L. amazonensis*, is a partially self-complementary ODN. Here we have shown that such an ODN (SBC 25Le) is an efficient inhibitor of *in vitro* translation, whereas the same ODN with unmodified bases (NB 25Le) is without activity. We presume that stable hairpin formation by the NB ODN prevents it from interacting with the mini-exon.

SBC ODNs with 2-aminoadenine and 2-thiophosphate bases might favor the formation of A-type double helices upon binding to RNA, leading to very stable hybrids. Each of the modified bases used here projects a bulky group into the minor groove of the duplex. The wider minor groove of the A-form duplex should better accommodate these groups. Previous studies of ODNs that contain 2-aminoadenine or 2-thiophosphate indicate a propensity to form A-type duplexes (23, 31, 32). The SBC ODNs used here have a high proportion of both modified bases and most likely share this tendency.

The retention of RNase H susceptibility by SBC ODN-RNA hybrids implies that the presence of the bulky groups in the minor groove does not prevent recognition of the duplex as a substrate by RNase H. This property of SBC oligos is unusual since other ODNs that form A-type hybrids with RNA, such as ODNs with 2'-O-alkyl or phosphoramidate backbones, do not allow RNase H activity (33, 34). The ability of NB and SBC ODNs with a PS backbone to support RNase H activity is consistent with the hypothesis that the presence of a β-anomeric deoxyribose as part of a negatively charged backbone allows the complementary RNA strand to be cleaved. However, the different cleavage patterns observed suggest that RNase H is able to sense the minor conformation changes induced by the presence of the modified bases.

Phosphorothioate ODNs were shown to exhibit *L. amazonensis*-killing activity in murine macrophages infected with an amastigote inoculum. The results obtained here with NB/16Le phosphorothioate are in good agreement with a previous study (8). The SBC ODNs exhibited a limited increased leishmanicidal activity compared with regular phosphorothioate ODNs. However, it should be noted that the maximal expected leishmanicidal effect was reached at a low concentration of the paired SBC 15-mers (15Le-I + 15Le-II); 30% cured macrophages were observed at 5 μM mixture (*i.e.*, 2.5 μM amounts of each oligomer). One cannot exclude the possibility that in live parasites the mini-exon sequence on the mature RNA does not fold into the hairpin. Sequestration of these PS ODNs by cellular proteins might also have partly negated the benefit of their increased affinity for the target (26). The additional thio groups contributed by 2-thiophosphate bases in PS SBC ODNs may enhance adsorption onto proteins.

Like the mini-exon sequence, numerous sequences in many transcripts are not freely accessible to complementary antisense ODNs due to their participation in secondary or tertiary structures (35–37). Efforts to overcome secondary structure by designing ODNs with greater binding affinity or by utilizing hybridization strategies that accommodate pre-existing RNA structure have not been entirely successful (for recent overviews, see Refs. 15, 17, and 38). The unequivocal advantage of SBC 25Le argues the merit of testing whether paired sense-antisense SBC ODNs show a similar advantage in targeting random mRNA sequences; by co-administering a single-stranded antisense ODN and its complement, with SBC character, any secondary structure involving the site of interest could be disrupted by concurrent binding of both single-stranded SBC ODNs to the RNA sequences involved in base pairing.

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### REFERENCES

1. Toulmé, J. J. (1992) *in Antisense RNA and DNA* (Murray, J. A. H., ed) pp. 175–194, Wiley Inc., New York.
2. Toulmé, J. J., Bourget, C., Compagno, D., and Yurchenko, L. (1997) *Parasitology* 114, 545–559.
3. Borst, P. (1986) *Annu. Rev. Biochem.* 55, 701–732.
4. Cornelissen, A. W. C. A., Verspieren, P., Toulmé, J. J., Swinkels, B. W., and Borst, P. (1986) *Nucleic Acids Res.* 14, 5605–5614.
5. Byard, J. A., Eder, P. S., Engman, D. M., Brentano, S. T., Walder, R. Y., Knouton, D. S., Durman, D. M., and Donelson, J. E. (1986) *Science* 235, 569–571.
6. Pascolo, E., Blonski, C., Shire, D., and Toulmé, J.-J. (1993) *Biochimie* 75, 43–47.
7. Verspieren, P., Cornelissen, A. W. C. A., Thong, N. T., Héline, C., and Toulmé, J.-J. (1987) *Gene* 56, 307–315.
8. Ramazelle, C., Mishra, R. K., Moreau, S., Pascolo, E., and Toulmé, J. J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 7859–7863.
9. Mishra, R. K., Moreau, C., Ramazelle, C., Moreau, S., Bonnet, J., and Toulmé, J. J. (1995) *Biochim. Biophys. Acta* 1264, 229–237.
10. Bruzik, J. P., Van Doren, K., Hirsch, D., and Steitz, J. A. (1988) *Nature* 335, 559–562.
11. Lecuyer, K., and Crothers, D. M. (1993) *Biochemistry* 32, 5301–5311.
12. Harris, K. A., Crothers, D. M., and Ulba, E. (1995) *RNA* 1, 351–362.
13. Verspieren, P., Loreau, N., Thong, N. T., Shire, D., and Toulmé, J. J. (1999) *Nucleic Acids Res.* 18, 4711–4717.
14. Pascolo, E., Hudriska, D., Spessat, B., Thong, N. T., and Toulmé, J.-J. (1994) *Biochim. Biophys. Acta* 1219, 88–106.
15. Toulmé, J.-J., Le Tinévé, R., and Brossalina, E. (1996) *Biochimie* 78, 663–673.
16. Pascolo, E., and Toulmé, J.-J. (1996) *J. Biol. Chem.* 271, 24187–24192.
17. Kutyavin, I. V., Rhinehart, R. L., Lukhtanov, E. A., Gorn, V. V., Meyer, R. B.,
and Gamper, H. B. (1996) Biochemistry 35, 11170–11176
18. Woo, J., Meyer, R., and Gamper, H. B. (1996) Nucleic Acids Res. 24, 2470–2475
19. Iyer, P. I., Lawrence, R. P., Egaz, W., Regan, J. B., and Beaucage, S. L. (1996) J. Org. Chem. 61, 4693–4698
20. Gamper, H. B., Reed, M. W., Cox, T., Viroso, J. S., Adams, A. D., Gall, A. A., Scholler, J. K., and Meyer, R. B., Jr. (1993) Nucleic Acids Res. 21, 145–150
21. Cantor, C., Warshaw, M. M., and Shapiro, H. (1970) Biopolymers 9, 1059–1077
22. Cheong, C., Tinoco, I., and Chollet, A. (1988) Nucleic Acids Res. 16, 5115–5122
23. Connolly, B. A., and Newman, P. C. (1989) Nucleic Acids Res. 17, 4957–4974
24. Bangs, J. D., Crain, P. F., Hashizume, T., McCluskey, J. A., and Boothroyd, J. C. (1992) J. Biol. Chem. 267, 9805–9815
25. Howard, F. B., and Miles, H. T. (1984) Biochemistry 23, 6723–6732
26. Cazenave, C., Loreau, N., Thuong, N. T., Toulmé, J. J., and Hélène, C. (1987) Nucleic Acids Res. 15, 4717–4736
27. Boizau, C., Kurfurst, R., Cazenave, C., Roig, V., Thuong, N. T., and Toulmé, J. J. (1991) Nucleic Acids Res. 19, 1113–1119
28. Cazenave, C., Frank, P., and Büsken, W. (1995) Biochimie 75, 113–122
29. Monia, B. P., Lesnik, E. A., Gonzalez, C., Lima, W. F., Mcgee, D., Guinosso, C. J., Kawasaki, A. M., Cook, P. D., and Freier, S. M. (1993) J. Biol. Chem. 268, 14514–14522
30. Newman, P. C., Nwosu, V. U., Williams, D. M., Cosstick, R., Seela, F., and Connolly, B. A. (1990) Biochemistry 29, 9891–9901
31. Garriga, P., Garcia-Quintana, D., Sagi, J., and Manyosa, J. (1993) Biochemistry 32, 1067–1071
32. Cazenave, C., Frank, P., and Büsken, W. (1995) Biochimie 75, 113–122
33. Inoue, H., Hayase, Y., Iwai, S., and Ohtsuka, E. (1987) FEBS Lett. 215, 327–330
34. DeDionisio, L., and Gryaznov, S. M. (1995) J. Chromatogr. B 669, 125–131
35. Chastain, M., and Tinoco, I., Jr. (1993) in Antisense Research and Applications (Crooke, S. T., and Lebleu, B., eds) pp. 55–66, CRC Press, Boca Raton, FL
36. Laptev, A. V., Lu, Z. C., Colige, A., and Prockop, D. J. (1994) Biochemistry 33, 11033–11039
37. Uhlenbeck, O. C. (1995) RNA 1, 4–6
38. Ecker, D. J. (1993) in Antisense Research and Applications (Lebleu, B., and Crooke, S. T., eds) pp. 387–399, CRC, Boca Raton, FL
39. Miller, S. I., Landfear, S. M., and Wirth, D. F. (1986) Nucleic Acids Res. 14, 7381–7386