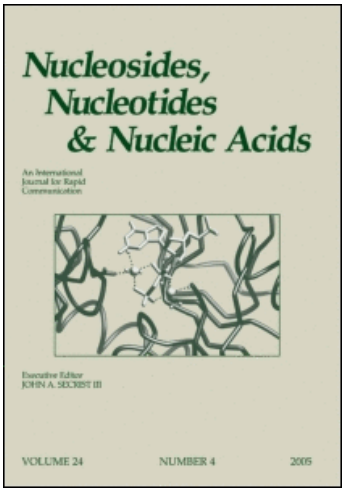

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Effect of Antisense Oligonucleotides Linked to Alkylating Agents on In Vitro Translation of Rabbit β-Globin and *Typuaosomu brucei* mRNAs

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To cite this Article Boiziau, C. , Boutorine, A. S. , Loreau, N. , Verspieren, P. , Thuong, N. T. and Toulmé, J. J.(1991) 'Effect of Antisense Oligonucleotides Linked to Alkylating Agents on In Vitro Translation of Rabbit β-Globin and *Typuaosomu brucei* mRNAs', Nucleosides, Nucleotides and Nucleic Acids, 10: 1, 239 — 244
To link to this Article: DOI: 10.1080/07328319108046451
URL: http://dx.doi.org/10.1080/07328319108046451

NUCLEOSIDES & NUCLEOTIDES, 10(1-3), 239-244 (1991)

Effect of antisense oligonucleotides linked to alkylating agents on in vitro translation of rabbit β-globin and *Trypanosoma brucei* mRNAs.

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Introduction

Antisense oligonucleotides have been used to artificially regulate the expression of numerous genes, both in vitro and in vivo (1). Studies in cell-free extracts and in micro-injected frog oocytes have shown that oligomers complementary to the coding region cannot prevent polypeptide synthesis by the elongating ribosome, unless the RNA part of the oligonucleotide/RNA hybrid is degraded by RNase-H (2,3). Consequently, oligomers targeted downstream from the AUG initiation codon that do not elicit RNase-H activity do not inhibit protein synthesis. This was demonstrated to be the case for methylphosphonate and alpha derivatives, which are resistant to degradation by nucleases (4,5).

Oligonucleotides which modify irreversibly the target RNA represent a new class of antisense oligomers. Such oligomers are expected to prevent polypeptide chain elongation. Various reactive derivatives can be linked to the oligonucleotide moiety:

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metal complexes, photosensitizers, etc... (6). Alkylating reagents, covalently linked to oligonucleotides have been used to modify single-stranded and double-stranded nucleic acids (7,8). We used α- and β-oligomers, bearing an aromatic nitrogen mustard, as antisense oligonucleotides against the coding region of the rabbit β-globin mRNA or the 5' leader region (so-called mini-exon sequence) which is present at the end of every mRNA from the protozoan parasite *Trypanosoma brucei* (9).

Material and Methods

A conventional 16-mer, complementary to nucleotides 7-22 of the mini-exon sequence of *Trypanosoma brucei,* was synthesized on an Applied Biosystems 380B synthesizer. The region 113-129 of the rabbit β-globin mRNA was chosen as a target for two 17-mers prepared either with β- (17β) or with α-anomers (17α) of nucleoside units. From previous studies, the α-oligomer was designed to bind in a parallel orientation to the target RNA (4). Oligonucleotides were then 5' phosphorylated with T4 polynucleotide kinase (in the presence of [^{32}P]γATP), purified from residual ATP by gel filtration and visualized by autoradiography after electrophoresis. The alkylating reagent [4(N-2-chloroethyl-N-methyl)amino-benzyl]methylamine, was linked to the 5' end of phosphorylated oligomers according to a previously published procedure (10). Modified oligonucleotides 16β-Alk, 17β-Alk and 17α-Alk were purified by reverse phase HPLC on a C_{18} Interchim column with a 5-40% acetonitrile gradient in 10mM ammonium acetate buffer, pH 7.2.

Trypanosomes [clone 118a] of *Trypanosoma brucei brucei* were cultured on SDM79 growth medium containing 10% fetal calf serum. For experiments with oligonucleotides, cells were resuspended at 10^6 parasites/ml in 100µl of SDM79 containing the oligonucleotide at the indicated concentration. Incubation was performed at 26°C with gentle shaking, aliquots were withdrawn and cells counted.

Mini-exon derived (med) RNA and total RNA from cultured forms of *T. brucei* were prepared according to previously published procedures (11,12). Rabbit globin mRNA was purchased from Bethesda Research Laboratory. For covalent binding rabbit globin or trypanosome mRNA were incubated for two hours at 35°C, in the presence of a 150-fold excess of oligonucleotides 16β-Alk, 17β-Alk and 17α-Alk, in a 10mM cacodylate buffer pH 6.8 containing 50mM NaCl. Med-RNA from trypanosomes was analysed by electrophoresis on a 8%polyacrylamide/7M urea gel eventually followed by Northern blotting.

Rabbit reticulocyte lysate and wheat germ extract were purchased from Genofit (Geneva). Modified or unmodified mRNA (100ng rabbit globin mRNA or 2µg trypanosome RNA) was added to the translation mixture containing amino acids (including 15µCi of [^{35}S]-methionine, Amersham). The rabbit β-globin mRNA concentration was 7.8 and 9.4 nM in the wheat germ and in the reticulocyte lysate, respectively. Translation was allowed to proceed either for 1h at 25°C (wheat germ) or for 45mn at 30°C (reticulocyte lysate). Labelled proteins were then analysed by precipitation with trichloroacetic acid or by electrophoresis on 15% polyacrylamide gel containing 0.15% Bis acrylamide, 8mM Triton X100, 6M urea and 5% acetic acid (2).

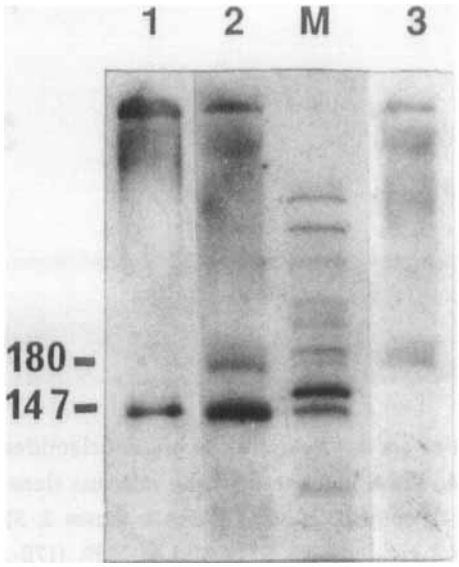

Figure 1: Cross-linking of 16β-Alk to *Trypanosoma brucei* med-RNA. RNA samples incubated in the absence (lane 1) or in the presence of unlabelled 16β-Alk (lane 2) or of ^{32}P-labelled 16β-Alk (lane 3), as described in Material and Methods, were run on a denaturing polyacrylamide gel. Samples corresponding to lanes 1 and 2 were transferred to a nylon membrane. The blot was probed with a 20-mer complementary to nucleotides 43-62 of med-RNA. The adduct formed by ^{32}P-labelled 16β-Alk was visualized directly by autoradiography (lane 3). Lane M: size markers.

Results and Discussion

Incubation of RNA with complementary alkylating oligonucleotides resulted in the specific cross-linking of the two nucleic acids. Results obtained with 16β-Alk and med-RNA are shown on Figure 1. The reaction product migrated as a single band corresponding to a fragment with an apparent mobility of about 180 nucleotides (lane 3) compared to the 140 nucleotide long med-RNA piece (lane 1). Northern blot analysis revealed the presence of two bands corresponding to the unreacted RNA and to the 16β-Alk/med-RNA adduct (lane 2). Densitometer tracing of the autoradiograph indicated that the yield of the reaction was about 40%. In the case of globin mRNA, about 95% and 50% of the β-globin RNA was cross-linked to 17β-Alk and 17α-Alk, respectively (not shown).

Translation of rabbit globin mRNA in wheat-germ extracts led to the synthesis of α- and β-chains of hemoglobin (Figure 2). When the extract was programmed with mRNA reacted with 17α-Alk, this resulted in the specific inhibition of β-globin synthesis (lane 1). The extent of inhibition (about 50%) was consistent with the amount of cross-links. In vitro translation of globin mRNA pre-treated with 17β-Alk

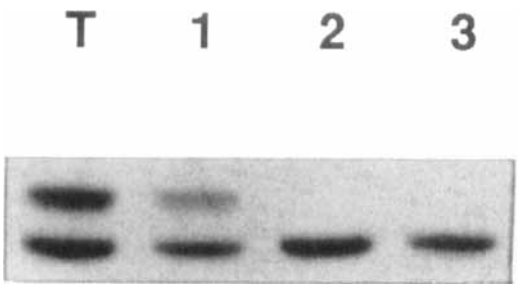

Figure 2: Effect of alkylating antisense oligonucleotides on in vitro translation of rabbit globin mRNA. RNA incubated in the absence (lane T) or in the presence of 1μM 17α-Alk (lane 1), or of 0.75μM of 17β-Alk (lanes 2, 3) was translated in wheat germ extract. Reaction yielded 50% (17α-Alk) or 100% (17β-Alk) cross-linked β-globin mRNA. The RNA reacted with 17β-Alk was translated in the absence (lane 3) or in the presence of an equal amount of unmodified RNA (lane 2). ^{35}S-labelled β- (upper band) and α-globin (lower band) were analyzed as described in Material and Methods.

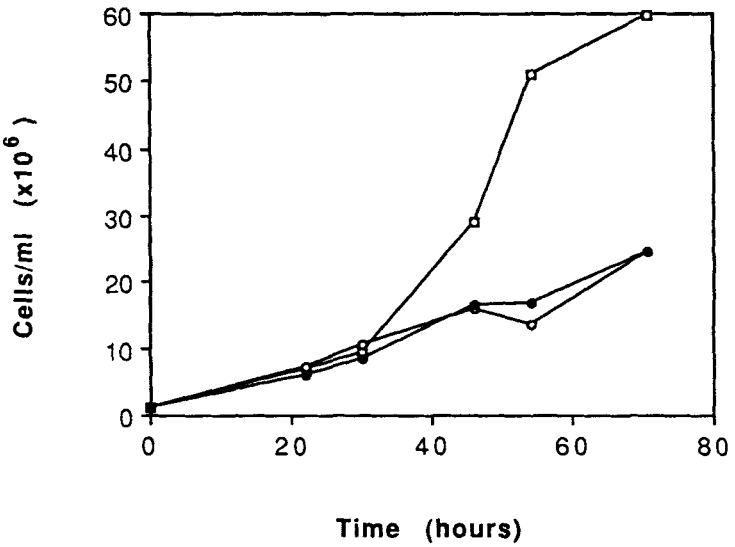

Figure 3: Effect of alkylating oligonucleotides on the growth of *Trypanosoma brucei*. 16β-Alk(○) or 17β-Alk (●) were added (0.5μM final concentration) to a culture of parasites in SDM79 growth medium. The curve (□) corresponds to trypanosomes grown in the absence of oligonucleotide.

reduced the β-globin synthesis to zero even with an RNA sample containing 50% (lane 3) cross-linked target. The different behavior exhibited by 17α-Alk and 17β-Alk is related to the sensitivity of the oligonucleotide/RNA hybrids to RNase-H. Binding of 17β induces the cleavage of the β-globin mRNA by wheat germ RNase-H, which subsequently results in translation inhibition. In contrast 17α does not elicit RNase-H degradation of the associated RNA and does not display any antisense property (4). Therefore, linking the oligomer to its complementary RNA sequence via alkylation reaction converted the inefficient α-oligonucleotide into a powerful antisense molecule. Cleavage of 17β-Alk/RNA hybrid by RNase-H led to a higher inhibitory efficiency of this oligomer compared to its α-congener.

We performed in vitro translation, in rabbit reticulocyte lysate, of trypanosome mRNA reacted with the oligonucleotide 16β-Alk. As the mini-exon sequence is present at the 5' end of every trypanosome mRNA, it was not possible to monitor the formation of cross-linked products. Under the same reaction conditions, about 40% of med-RNA was cross-linked (see above). In contrast to what was observed with globin mRNA, the antisense oligomer bearing the alkylating group was not more efficient than the unmodified oligonucleotide (not shown). This discrepancy might be due to different reasons. First these oligomers are targeted to a region close to the cap site. In this region antisense oligonucleotides exert their inhibitory activity through an RNase-H independent mechanism: they prevent the binding of the initiation complex (5, 13; Boiziau et al., unpublished results). Second, the mini-exon region might adopt different secondary structures in med-RNA and in mature mRNA. Secondary structures might interfere with the formation of oligonucleotide/RNA adducts.

We then investigated the effect of 16β-Alk on intact parasites in culture. As shown in Figure 3 the presence of this oligomer in the growth medium reduced the development of parasites. However a similar behaviour was observed with 17β-Alk which is not complementary to the mini-exon sequence. Therefore the decreased growth rate very likely originates in a non-specific effect of alkylating oligonucleotides. No effect was observed with the homologous unmodified 16-mer at the same concentration.

Conclusion

Our results with the rabbit β-globin gene demonstrate that protein synthesis can be significantly inhibited by antisense oligonucleotides that introduce irreversible modification of the target sequence. Despite their inability to induce the cleavage of the target RNA sequence α-oligonucleotides cross-linked to their target RNA sequence blocked translation in cell-free extracts. This will allow to fully take advantage of the long lifetime of α-oligomers in the presence of DNases (growth media, living cells). Similar results were obtained with oligonucleoside methylphosphonate analogues linked to a psoralen derivative (14). However, in our case, cross-linking did not require light activation. Adducts with poly(A) tails of

mRNA have previously been characterized in Krebs ascite tumor cells (15). Therefore, alkylating oligonucleotide derivatives could be interesting tools for studies with cultured or micro-injected cells.

Acknowledgements

We thank Dr V.P. Starostin and T.M. Ivanova for a gift of [4(N-2-chloroethyl-N-methyl)amino-benzyl]methylamine. This work was supported by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. A.S. B. was supported by INSERM.

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