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OLIGODEOXYRIBONUCLEOTIDE PHOSPHOROTHIOATES KILL PROCYCLIC *TRYPANOSOMA BRUCEI BRUCEI* : QUANTITATIVE DETERMINATION OF THEIR LD_{50}.

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Abstract: Phosphorothioates kill the procyclic form of *T. brucei brucei* by a non antisense but sequence dependent effect. The alamar Blue™ method allowed an easy microscale determination of their antiparasitic effect. The LD_{50} of the sequences tested was in the range of 11-20 μM. © 1997 Elsevier Science Ltd.

Trypanosomes are protozoa responsible for life-threatening endemic diseases. Sleeping sickness is produced by *T. brucei gambiense* and *T. brucei rhodesiense*. *T. brucei brucei* is not infective for humans but causes bovine trypanosomiasis, called nagana. There is a real need to develop new trypanocidal compounds because the few drugs that are nowadays available do not always guarantee cure, may cause severe side-effects or are not all active in the late (cerebral) stage of the disease[1]. Antisense oligonucleotides could be effective trypanocides, at least at the beginning of the infection, when the parasite is still directly accessible in the blood. Verspieren *et al.*[2] were the first to consider this strategy, using an oligodeoxyribonucleotide coupled to acridine at its 3'-end whose target was the mini-exon, a sequence present at the 5'-end of all the trypanosomal mRNA's. At a concentration of 130 μM this modified oligonucleotide killed the procyclic parasites in 45 h. It was without effect at concentrations lower than 80 μM and the culture medium had to be deprived of serum to enhance its efficacity, owing to its digestion by seric enzymes. In cell free systems, long anti mini-exon oligonucleotides and relatively high concentrations were necessary to inhibit the translation of trypanosomal mRNA's[3,4]. We thus decided to target not a mRNA, but another sequence, a snRNA. Tschudi and Ullu[5], using detergent permeabilized trypanosomes, demonstrated that a pentadecameric oligonucleotide, complementary to the nucleotides 31-45 of the U2 snRNA, abolishes trans splicing[6]. Palfi *et al.*[7] were able to isolate, by affinity chromatography, the U2 snRNP of *T. brucei*, using the same sequence immobilized on streptavidin-agarose. We thus selected as potential trypanocides the oligomers **1** and **2**, complementary to nucleotides 31-45 and 27-46 of the trypanosome U2 snRNA, because the targeted region is accessible and hybridization at this site apparently interferes with splicing. Moreover, since there are large differences between this part of the trypanosomal U2

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snRNA and the human one, selective toxicity could be expected. The sequences of interest are reported in the table[8].

TABLE : TARGET AND TESTED SEQUENCES

Trypanosomal U2 snRNA, nucleotides 27-46
UCA AGU UAU UAA ACU GUU CU

Human U2 snRNA, nucleotides 27-46
UCA AGU GUA GUA UCU GUU CU

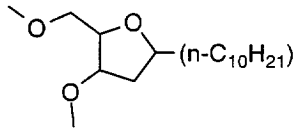
1 oligonucleotide phosphodiester complementary to the U2 snRNA
d(C AGT TTA ATA ACX XG)

2 oligonucleotide phosphorothioate complementary to the U2 snRNA
S-d(AG AAC AGT TTA ATA ACT TGA)

3 Control: scrambled **2**
S-d(TA AAC TAA ATA TGG TAG CAT)

4 S-d(C_{28})

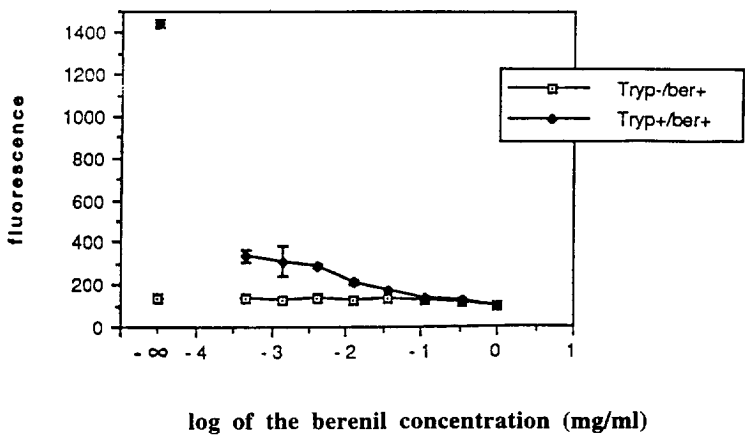
5 S-(T_{20})

X = 

The procyclic form of *T. brucei brucei* (stock 427) was cultivated in 96-well microtiter plates at 27°C using the SDM-79 medium, supplemented with 10 % (vol/vol) fetal calf serum (heat-inactivated at 56°C for 30 min.) and 5 mg of hemin per liter. The parasites were introduced at a concentration of 10^5 cells/ml and were allowed to multiply for 72 h in the presence or absence of effectors. At this time, the cultures were observed at the invertoscope and the living trypanosomes were titrated by the alamar Blue™ method[10].

FIGURE 1

Black dots: effect of increasing concentrations of the trypanocide berenil on the fluorescence of alamar Blue™ 72 h after the beginning of the culture of *T brucei brucei*. White dots (blank): increasing concentrations of berenil had no effect on the fluorescence of alamar Blue alone.



Since the titration of procyclic trypanosomes by this technique has not yet been described before, it had to be evaluated. Figure 1 shows the fluorescence at 72 h of the cultures in the absence or in the presence of increasing amounts of berenil, an established trypanocide. Trypanosomes were completely destroyed by a concentration of 0.1 mg/ml (194 μM) of berenil. The residual value of the fluorescence for the killed culture was the same as that of the blank without parasites. The inspection at the invertoscope confirmed the disappearence of the trypanosomes. We then tested our oligonucleotides.

Oligonucleotide **1** had no effect on the cultures (not shown). This cannot be ascribed to a rapid digestion by the serum enzymes as **1** is resistant, even to undiluted (unheated) fetal calf serum[8]. However, a digestion by the ecto-enzymes of the parasite during the process of cellular penetration cannot be excluded. Trypanosomatidae cannot synthesize purines *de novo*. A very effective system to capture purines from the external medium has been described for these parasites, but it uses nucleotide 3'-phosphates or fragments of RNA as substrates, not fragments of DNA. An ecto-3'nucleotidase/nuclease liberates the nucleosides that are then transported into the cytosol. The enzyme is unable to dephosphorylate 3'-deoxyribonucleotides[11-14]. The 2'-oxy versus deoxy selectivity is probably due to an involvement of the 2'-OH in the mechanism of hydrolysis. Besides this ecto-3'-nucleotidase/nuclease, procyclic *T. brucei brucei* also possesses an acid ecto-phosphodiesterase of unknown substrate specificity[15]. This last enzyme could possibly digest oligodeoxyribonucleotides. Verspieren et al.[2] and Boiziau et al.[16] also reported that simple anti mini-exon phosphodiester oligonucleotides are without effect on *T. brucei brucei*. We thus decided to try phosphorothioates, that are known to be far more resistant to hydrolysis.

Oligonucleotides **2**, **3** and **4** were able to kill the parasites. Panels A-C of figure 2 show that the oligonucleotide having as target the U2 snRNA **2** as well as the corresponding scrambled sequence **3** or S-d(C_{28}) **4** were practically equipotent. The homo-oligomer S-d(C_{28}) was used in this study, because it is known to have a high affinity for proteins situated on the surface of eukaryotic cells[17,18]. S-(T_{20}) was not toxic for the trypanosomes in the concentration range studied (panel D).

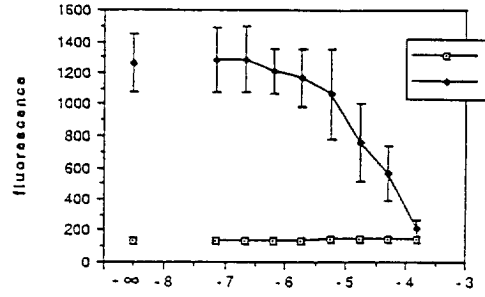
Although the expected antisense effect was not observed, the LD_{50} of the tested oligomers (the LD_{50} was the concentration that reduced the fluorescence to half its initial value as seen in figure 2, i. e. 11, 11 and 20 μM for **2**, **3** and **4**, respectively) was lower than that of the best antisense molecules active on *T. brucei brucei* previously reported (LD_{50} higher than 80 μM, without serum)[2]. It was also reported that anti mini-exon phosphorothioates were without effect on the promastigote form of another trypanosomatidae, *Leishmania amazonensis* [19].

Oligonucleotides are polyanions that do not easily penetrate into the cytosol of eukaryotic cells[20]. They are effectively endocyted but a large amount remains sequestred into reservoir endosomes[17,18]. The non-antisense effect we observe here could be due to the interaction of the phosphorothioates with proteins of the flagellar pocket before and/or during endocytosis. The effect is sensitive to the sequence, as S-(T_{20}) proved to be non toxic. The observed sequence sensitivity justifies the search for phosphorothioates active at lower concentration. Non-antisense inhibition on the growth of another protozoan, *Plasmodium falciparum* was detected at concentrations as low as 1 μM[21]. A random search for an optimal sequence by combinatorial automatic synthesis would be feasible for biopolymers such as oligonucleotides. An easy method of screening is however essential. The classical screening on microtiter plates by the counting of surviving *T. brucei brucei* cells was rendered difficult by the aggregation of the parasites when treated with our trypanocidal agents. It is not unusual that surviving trypanosomes adhere to congeners in a progressive state of lysis, as observed by Coppens *et al.* in another context[22].

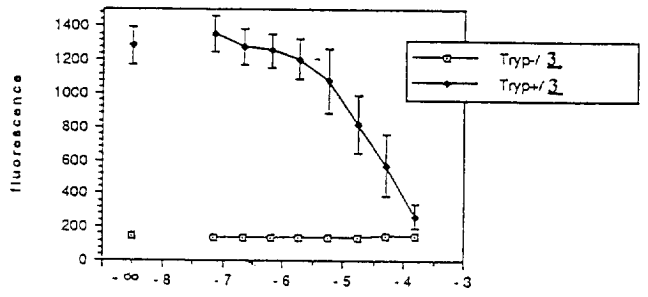
FIGURE 2

Black dots: effect of increasing concentrations of oligonucleotides 2-5 on the fluorescence of alamar Blue™ 72 h after the beginning of the culture of *T. brucei brucei*. White dots (blank): increasing concentrations of oligonucleotides 2-5 had no effect on the fluorescence of alamar Blue alone. The curves correspond to the mean of two serial dilutions on the same microtiter plate. A repetition of the experiment gave the same results.

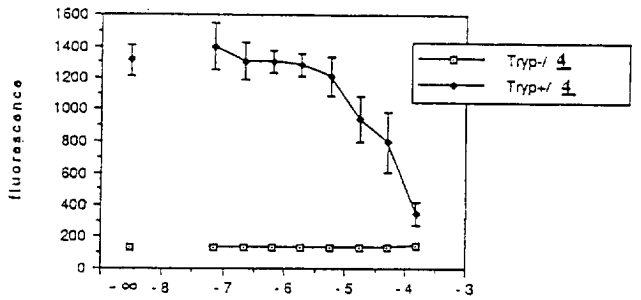
Panel A



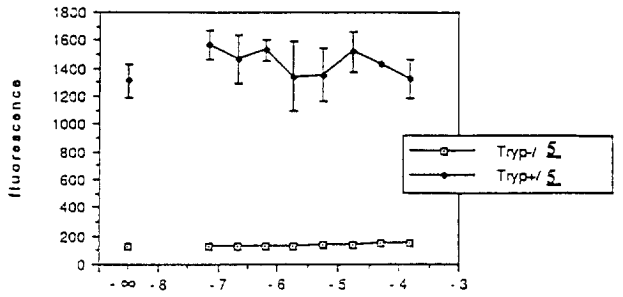
Panel B



Panel C



Panel D



log of oligonucleotide concentrations (mole/l)

Preliminary results have shown that the alamar Blue™ method also works with the bloodstream form of *T. brucei* in the axenic culture conditions of Hirumi and Hirumi[23]. The use of an easy biochemical test to quantitate living trypanosomes thus renders the search for an optimal trypanocidal sequence feasible.

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6. The sole concentration tested was high (200 µg/ml, 44 µM) because the aim of the work was to decifer the role of the various small ribonucleoproteins in the splicing, not to search for the minimum inhibitory concentration.
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8. The oligonucleotides were synthesized with a Pharmacia LKB gene Assembler Plus. The sulfurization was performed with Beaucages' reagent. The oligomers were purified by reversed phase HPLC (BIOSIL C_{18} HL 90 SS BIORAD), followed by gel exclusion chromatography (NAP 10 DNA grade, Pharmacia) or, alternatively, by a gel exclusion chromatography followed by ethanol precipitation. The oligonucleotides purified by either method had the same biological activities. The ethanol precipitation was preferred because the reversed phase column tended to be irreversibly contaminated by the phosphorothioates. The homogeneity and the size of the oligonucleotides were checked by PAGE. Oligomer **1** featured two hydrophobic residues at the penultimate position of the 3' end, in order to block the digestion by 3' exonucleases and enhance the adsorption on the cellular surface[9]. The protection against exonucleases was effective as the sole modification of **1** after staying 24 h in neat (not heated) fetal calf serum was the loss of the terminal p-d(G) nucleotide. The control oligonucleotide d(C AGT TTA ATA AC) was totally digested in the same sample of serum after 12 h. Snake venom 3' phosphodiesterase (Crotalus Adamanteus, Pharmacia) also removed the terminal p-(dG) but did not progress further along the sequence.
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10. Alamar Blue (ACCUMED) is a redox indicator whose fluorescence in the reduced state allows to detect living cells. It is minimally toxic to cellular cultures. After uptake by the cells, alamar Blue is reduced by cellular oxidoreductases. The solution of alamar Blue (10 µl) was added to the cultures (100 µl) at 72 h

and the incubation was pursued for 4 h (27°C) before the fluorescence reading. A Cytofluor Millipore 2300 apparatus was used with an exciting wavelength of 530 nm and an emission wavelength of 590 nm.

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