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Maria Vega C. Flores · David Atkins · Thomas Stanley Stewart · Arthur van Aerschot · Piet Herdewijn

Antimalarial antisense activity of hexitol nucleic acids

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Abstract Antisense oligonucleotides and ribozymes have shown promise both as antimalarial agents and as tools for identifying genes vital for parasite survival. This task is urgent due to the ineffectiveness of current drug regimes on the most virulent human malarial parasite, *Plasmodium falciparum*. The development of new ways to modify and/or protect conventional phosphodiester oligonucleotides to improve nuclease resistance is also important. We assessed the effect of antisense oligonucleotides containing phosphorylated anhydrohexitols in suppressing the growth of *P. falciparum* in culture. The modified oligonucleotides were able to inhibit parasite growth in a sequence-specific manner, but not as well as the phosphorothioated antisense oligonucleotides, which are effective antimalarials at submicromolar concentrations. Two reasons are suggested: the absence of RNase H activation and differences in membrane transport.

Introduction

Malaria remains one of the major infectious diseases of humankind and has been resurgent in recent years because of the failure of control procedures including previously effective chemotherapy. Approaches to limit the spread of the disease include the development of an effective vaccine, parasite vector control and the search for novel chemotherapies (Butler 1997). Recent studies have explored the suitability of using nucleic acids as antimalarial agents. Promising results have been obtained with both antisense DNA (Barker et al. 1996, 1998; Katrib et al. 1997) and synthetic ribozymes (Flores et al. 1997; M.V.C. Flores, D. Atkins, T.S. Stewart, unpublished data). As with most applications of synthetic nucleic acids, a key limitation is the biological stability of the active agents. Considerable effort has been put into modifing oligonucleotides to introduce RNAse-resistant internucleotide linkages. This report describes the study of antisense oligonucleotides containing a phosphorylated anhydrohexitol backbone structure (HNA). It has been demonstrated previously that HNAs hybridise sequence selectively and very strongly with complementary RNA sequences (Hendrix et al. 1997). In addition, HNAs are stable against enzymatic degradation. As HNA-RNA duplexes do not activate RNase H, the antisense effect of HNA may be attributed to a steric block of the target RNA. The antisense HNA sequences targetted the carbamoyl phosphate synthetase II gene (pfCPSII; Flores et al. 1994) of the most virulent human malaria parasite, *Plasmodium falciparum*. The pfCPSII gene encodes the first and limiting enzyme in the vital pyrimidine biosynthetic pathway. Hammerhead ribozymes (Flores et al. 1997; M.V.C. Flores, D. Atkins, T.S. Stewart, unpublished data) and antisense DNA (Katrib et al. 1997) directed against this gene have been previously shown to effectively suppress malarial growth in culture.

M.V.C. Flores (✉) · T.S. Stewart
School of Biochemistry and Molecular Genetics,
The University of New South Wales, Sydney,
New South Wales, 2052, Australia
e-mail: m.flores@unsw.edu.au
Fax: +61-2-9385 1483

D. Atkins
Corporate Office for Science and Technology,
Johnson & Johnson, New Brunswick, NJ 08901-2021, USA

A. van Aerschot · P. Herdewijn
Laboratory for Medicinal Chemistry,
Raga Institute for Medical Research,
Minderbroedersstraat 10, B-3000, Leuven, Belgium

Materials and methods

Oligonucleotide sequences derived from the pfCPSII gene used for screening are listed in Table 1, with MH1–MH5 representing fully modified HNA (indicated with prefix h), MH6 and MH7 phosphorothioate (PS) oligodeoxynucleotides, while MH8 and MH9 comprise HNA sequences with a thioated deoxynucleotide stretch as window. All sequences were prepared on a 1,3-propanediol-

Table 1 Oligonucleotide sequences for the malaria inhibition assays

HNA oligos	Sequence	Nucleotide numbers
MH1	h(TCA TGT TCT TGA CCT TGA TAA-4′-Pr	3724–3744
MH2	h(CCT CAG TGC TGA CAG CCC ATC)-4′-Pr	Random sequence
MH3	h(CGT CTG AAC TAA ATT TTG CTT)-4′-Pr	Random sequence
MH4	h(GAA ATA TAC ATA AGA AAA ATG)-4′-Pr	−10 to 11
MH5	h(AAC TAA GAT AAA TAA GAT AGA)-4′-Pr	Random sequence
MH6	GAA ATA TAC ATA AGA AAA ATG-3′-Pr	−10 to 11
MH7	AAC TAA GAT AAA TAA GAT AGA-3′-Pr	Random sequence
MH8	h(TCA TGT)-TCT TGA CC-h(T TGA TAA)-4′-Pr	3724–3744
MH9	h(CGT CTG)-AAC TAA AT-h(T TTG CTT)-4′-Pr	Random sequence
Controls		
CPSM10	GAG ATA CTT GGA CAA TTA TTA	897–917
CPSM15	TCA TGT TCT TGA CCT TGA TAA	3724–3744
DHFR105	TCT TAA AAA TAA TTT CTT CGT AGT TAA	1153–1179

modified support (Pr), with the first nucleoside attached via a phosphodiester (thioate) linkage, endowing the sequences with higher nuclease resistance (Van Aerschot et al. 1995). Oligonucleotides used as reference controls have PS linkages and are antisense sequences to the pfCPSII gene in the case of CPSM10 and CPSM15 (Flores et al. 1994) and the *P. falciparum* dihydrofolate reductase gene in the case of DHFR105 (Bzik et al. 1987). CPSM10 and CPSM15 are the PS antisense versions (Katrib et al. 1997) of the ribozymes CPSRz1 anf CPSRz4, respectively.

The FCQ-27/Papua New Guinea (chloroquine-sensitive) strain of *P. falciparum* was grown in continuous culture in Type-O$^+$ human blood using a modified version of techniques described by Trager and Jensen (1976) as previously reported (Flores et al. 1991). The parasite strains were provided by the Army Malaria Research Unit, Ingleburn, Australia. Blood and serum were obtained from the Blood Bank Australia. Human serum was pooled from a minimum of ten donors, aliquoted and frozen until use.

Inhibition of synchronous ring-stage *P. falciparum* (Lambros and Vanderberg 1979) in culture was carried out after exposure of cells to oligonucleotides for 24 h as previously described (Flores et al. 1997). Initially, cell toxicity levels were checked with 0.5, 5, and 10 µ*M*, and in one case, 20 µ*M* oligonucleotide concentrations. Using our assay conditions, a series of PS antisense oligodeoxynucleotides inhibit parasite growth at 0.5 µ*M* by up to 40% (Katrib et al. 1997). Due to the differences in effective doses of the different oligonucleotides, two dilutions were prepared for each oligonucleotide before 'blinding' the tubes (Table 2).

The cultures were incubated for 24 h and the parasitaemia was determined as described earlier (Flores et al. 1997). All values presented are the average of at least three experiments for each oligonucleotide. Experiments were performed blind, and the 'code' was revealed following analysis.

Results and discussion

A summary of the bioassay on malarial parasite cultures for the test oligonucleotides is presented in Fig. 1. While malarial inhibition was detected using the PS antisense at concentration as low as 0.25 µ*M*, the effects of using the fully modified anhydrohexitols were only observed at concentrations higher than 10 µ*M*. Chimeric molecules composed of anhydrohexitols and phosphorothioate-linked DNA were effective at 5 µ*M*.

The HNA oligonucleotide MH1 targetting the sequence where the ribozyme CPSRz4 was effective (Flores et al. 1997) significantly reduced parasite growth (45%) compared to the random sequence controls, MH2 (5%) and MH3 (17%) at 20 µ*M*. A modified HNA oligonucleotide directed at the 5′ end of the pfCPSII mRNA around the ATG (MH4) only effected a 28% growth suppression. Our previous studies with mRNA folding as well as inhibition by ribozymes and antisense DNA show that the CPSRz4 site seems very accessible, and may facilitate oligonucleotide binding (Katrib et al. 1997). The 5′ end of the pfCPSII mRNA is very A + U rich and the mRNA-folding prediction shows it to be less accessible than further downstream. Similar unsat-

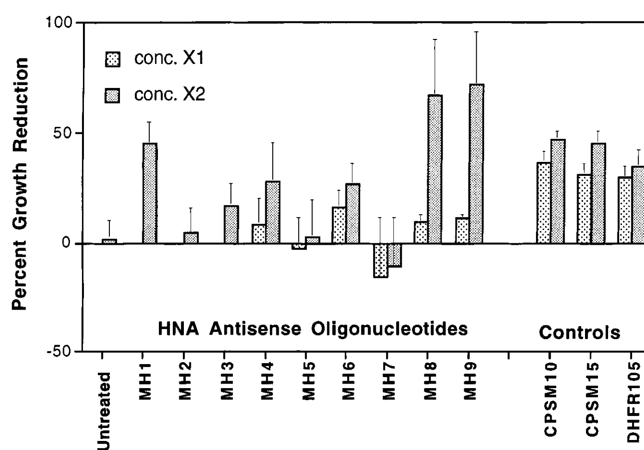

Fig. 1 Effect of HNA antisense oligonucleotides on the growth of *Plasmodium falciparum* in culture. Two concentrations of oligonucleotide (Table 2) were administered in triplicate parasite cultures at $t = 0$ to synchronised ring-form parasites. After 24 h incubation, the parasite cell count was determined, and the percent growth calculated from average parasitaemia in untreated controls. The difference from 100% (control average) is expressed as percent reduction on the y-axis. The data are means ± SD of three experiments, each with triplicate samples

Table 2 Oligonucleotide dilutions used for the bioassays

Oligonucleotide	Concentration ×1	Concentration ×2
MH1, MH2, MH3, MH4, MH5	10 µ*M*	20 µ*M*
MH6, MH7	0.5 µ*M*	1.0 µ*M*
MH8/MH9	5 µ*M*	10 µ*M*
CPSM10, CPSM15, DHFR105	0.5 µ*M*	1.0 µ*M*

isfactory predictions were shown for the 3′ end of the coding region of the gene. Indeed, PS oligonucleotides directed to these parts of the gene only afforded a 18–20% inhibition at 0.5 μ*M* compared to 37–40% for those directed at the CPSRz1 and CPSRz4 sites corresponding to CPSM10 and CPSM15 (Katrib et al. 1997), and as repeated in this study (Fig. 1). The lower effect of MH4, although sequence dependent, is likely to be dictated by the structure of the mRNA. This inhibition is sequence specific, as the random control sequence, MH5, gave a very minimal inhibition of 3% even at 20 μ*M*.

CPSM15, the PS antisense counterpart of MH1, was able to suppress malarial proliferation up to 37% at 0.5 μ*M* and 45% at 1.0 μ*M*. MH6 has the same sequence as MH4 but is composed of PS oligonucleotides, stabilised at the 3′ end with a propanediol phosphodiester. MH6 exerted 27% growth reduction at 1.0 μ*M*. These levels of inhibition are significant when compared to the negative controls and mimic the trend seen from the HNA oligonucleotides. However, the concentrations at which these thioate sequences are effective against malaria are almost 20 to 40-fold lower. This might be because cellular uptake levels of HNAs are lower than standard oligonucleotides. A second reason might be the difference in RNase H activation capabilities. Interestingly, the effective dose concentrations decreased from 20 to 5 μ*M* when part of the HNA was changed to PS DNA, indicating that these molecules may be taken up by the parasites more easily than the fully modified HNA oligonucleotides and/or might 'partially' function via an RNase-H-dependent mechanism in view of the presence of the window. MH8, the chimeric HNA and phosphorothioated oligonucleotide directed against the CPSRz4 region inhibited malarial growth by 67% at 10 μ*M*. However, this inhibition is seen to be non-specific as the negative control, MH9, likewise gave the same level of inhibition (72%). We assess this as a clear phosphorothioate effect especially at this very high concentration of oligonucleotides. It is very well documented in malarial inhibition assays that any phosphorothioated DNA at concentrations over 1.0 μ*M* increasingly produce non-sequence-specific effects (Barker et al. 1996).

In conclusion, HNAs demonstrate in vivo antimalarial effects, although they are less active than phosphorothioates. Unlike phosphorothioated DNAs, HNA/RNA hybrids do not activate RNase H, and could therefore reduce their anti-gene activity. The slight structural difference between natural 2′-deoxynucleosides and HNA may also reduce the intracellular uptake of the latter. However, HNAs form very stable complexes with DNA and RNA and are enzymatically stable (Van Aerschot et al. 1995). If minimal non-specific cellular toxicity, as seen in this study, can be maintained using HNAs, and the mechanism of membrane transport be improved, the usefulness of these molecules as antisense constructs will be enhanced.

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