Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 8577–8580, September 1992
Medical Sciences

Antimalarial activities of oligodeoxynucleotide phosphorothioates in chloroquine-resistant *Plasmodium falciparum*

(Malaria/drug resistance/modified oligodeoxynucleotides/antisense)

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Contributed by Paul Zamecnik, June 2, 1992

ABSTRACT **Synthetic oligonucleotides and their chemical modifications have been shown to inhibit viral and cellular gene expression by sequence-specific antisense hybridization to target mRNAs. We now report that oligodeoxynucleotide phosphorothioates and their nuclease-resistant modifications are effective in micromolar and submicromolar concentrations against the growth of both chloroquine-resistant and chloroquine-sensitive strains of *Plasmodium falciparum in vitro*. Parasitized human erythrocytes were found to be accessible to radioactively labeled oligodeoxynucleotides, whereas the uninfected erythrocytes did not permit any cellular entry of the same compounds. The dihydrofolate reductase-thymidylate synthase gene of *P. falciparum* was demonstrated to be a good target for sequence-dependent inhibition of plasmodial growth by exogenously administered modified oligonucleotides. The antimalarial activities observed *in vitro* were identical for chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*. The antimalarial activity of oligodeoxynucleotide phosphorothioates is related to sequence complementarity to certain regions of the plasmodial genome as well as to non-sequence-defined activities.**

Malaria, which is one of the most widespread of human pathogenic diseases, is resurging in Southeast Asia, Africa, and South America, mostly because of the spread of drug-resistant parasites (1). The mechanisms of drug resistance in *Plasmodium falciparum* are not fully understood and are related to the ability of resistant parasites to expel an accumulated schizontocidal agent such as chloroquine through specific channels (2). Efforts to develop new classes of antimalarial drugs that would circumvent drug resistance are underway.

Antisense oligonucleotides have been known for many years to exist in cells (3, 4), but their use as potential chemotherapeutic agents is of more recent vintage. Synthetic oligonucleotides administered exogenously compose an alternate class of therapeutic agents and have been demonstrated to inhibit the replication and expression of Rous sarcoma virus (5), human immunodeficiency virus (HIV) (6), and other viruses (7) or cellular proteins in *in vitro* or *in vivo* screening systems (8, 9). Chemical modifications of the internucleoside phosphate group of oligodeoxynucleotides to phosphoramidates or phosphorothioates (10, 11) have afforded reductions in the 50% inhibitory concentrations (IC_{50}) to the 0.1 μM range, largely because of the increased stability of the oligonucleotide, without significant effects on its base sequence-specific hybridization to target mRNAs. The sequence-specific inhibition is the result of hybridization of the exogenously added oligonucleotide and the cellular mRNA, yielding inhibition of translation and the associated activation of RNase H, which leads to accelerated degradation of the target mRNA.

Oligodeoxynucleotide phosphorothioates 18 or 21 nucleotides long composed of specific bases that are complementary to sequences of plasmodial mRNAs were synthesized and tested. Two of the genetic targets chosen for sequence-specific inhibition were part of the P195 gene, which codes for the protein precursor of three smaller proteins that are major surface antigens of merozoites and are thus required for the development of plasmodial merozoites (12). Merozoites are the form of the malarial parasite that breaks out of the erythrocyte and invades new erythrocytes (12, 13). These sequences included the first 21 nucleotides of the open reading frame starting with the AUG start codon (P195-I) and an 18-nucleotide sequence that codes for part of an alternate repeat of two tripeptide sequences occurring six and five times, respectively, in the protein sequence (P195-II). In addition, a target sequence on the malarial dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene (14) was also selected for the purpose of inhibiting the synthesis of these two proteins (DHFR and TS) by "antisense" treatment. The malarial parasite is unable to synthesize the purine ring *de novo* and has therefore to obtain preformed purine bases and nucleosides from the host erythrocyte by a salvage pathway. On the other hand, *P. falciparum* relies exclusively on the *de novo* synthesis of pyrimidines because of its inability to salvage preformed pyrimidine bases or nucleosides (15). Therefore, drugs that inhibit the *de novo* synthesis of pyrimidines are expected to be clinically effective against malaria. One such drug, pyrimethamine, which was shown by Hitchings and collaborators to inhibit protozoal dihydrofolate reductase to a much higher extent than mammalian host dihydrofolate reductase, is currently used against malaria in the clinic (16, 17). It is important to note, however, that drugs such as pyrimethamine are rendered less effective by the global emergence of resistant strains. The resistance of the parasite to this type of drug is due not only to mutations in the target genes leading to altered gene products (proteins) (18) but also to the acquired ability by the parasite to prevent the intracellular accumulation of a cytotoxic drug (2, 19).

MATERIALS AND METHODS

All oligodeoxynucleotides in Table 1 were synthesized on an automated DNA synthesizer (Biosearch 8700, MilliGen, Bedford, MA). The assembly of oligodeoxynucleotides and oligodeoxynucleotide phosphorothioates was performed by H-phosphonate chemistry (20), with purification and analysis as described (21). The strains of *P. falciparum* used for demonstrating the *in vitro* efficacy of oligonucleotides were W2, an Indochina clone exhibiting chloroquine resistance, and D6, a West African clone that is chloroquine sensitive.

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Abbreviations: DHFR-TS, dihydrofolate reductase-thymidylate synthase.

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Table 1. Chemical structure and target specificity of oligonucleotides tested as antimalarial agents

Name	Sequence	Chemical structure and target specificity
PSI	5′-TAA AAA GAA TAT GAT CTT CAT-3′	Oligodeoxynucleotide phosphorothioate complementary in sequence to the first 21 nucleotides of the ORF from the start codon of P195
PSII	5′-AGC AAC TGA GCC ACC TGA-3′	Oligodeoxynucleotide phosphorothioate complementary in sequence to the 18 nucleotide sequences in P195 coding for the first two tripeptide repeats
PNII	5′-AGC AAC TGA GCC ACC TGA-3′	Oligodeoxynucleotide phosphomorpholidate complementary in sequence to the same sequence in P195 as PSII
POII	5′-AGC AAC TGA GCC ACC TGA-3′	Oligodeoxynucleotide (phosphodiester internucleoside bond) complementary in sequence to the same sequence in P195 as PSII
PSIII	5′-GTC GCA GAC TTG TTC CAT CAT-3′	Oligodeoxynucleotide phosphorothioate having a sequence complementary to the first 21 nucleotides of the ORF of *P. falciparum* DHFR-TS gene starting with the start codon
PSNIII	5′-GTC GCA GAC TTG TTC CAT CAT-3′	Oligodeoxynucleotide phosphorothioate with the last 3′ phosphodiester bond being a phosphorbutylamidate for the inhibition of exonuclease activity, having the same sequence as PSIII
RI	5′-CTT GGC AGC TGC GCG TGA CAT-3′	Oligodeoxynucleotide phosphorothioate of mismatched sequence in which the 3′- end is complementary to the 5′-AUG-3′ translation start codon
RII	5′-AAA AAT ATT TAT TTT CTA A-3′	Oligodeoxynucleotide phosphorothioate of mismatched sequence
RIII	5′-CGC GGC GGC CCG CGG CGC CGG-3′	Oligodeoxynucleotide phosphorothioate of mismatched sequence

ORF, open reading frame.

Both strains were isolated at the Walter Reed Army Institute of Research (Washington). Strains were cultured by a modified version of the method of Trager and Jensen (22). Parasites were maintained in flasks under an atmosphere of 4% O_2/6% CO_2/90% N_2 in a 5–8% washed erythrocytic suspension in a complete medium composed of RPMI 1640 supplemented with 3 mg of TES sodium salt, 2 mg of glucose, 110 μg of sodium pyruvate, 300 μg of glutamine, 5 μg of hypoxanthine, and 25 μg of gentamycin per ml and with 10% (vol/vol) human plasma at 37°C. Both the erythrocytes and the plasma were fresh, of type A+, and were obtained from the American Red Cross. For comparative screening experiments involving a variety of treatments, parasitized erythrocytes (0.5%–1% parasitemia) were cultured in 48-well microculture plates (GIBCOware) at 5% hematocrit in a total volume of 1 ml per well. Parasitemia levels were determined by counting erythrocytes smeared on glass slides and stained with Diff-Quik Fix and Stain Set (Baxter Scientific Products, McGraw Park, IL). Parasites were classified according to their developmental stage as ring forms (without pigment; the first form after invasion of the erythrocyte by a merozoite), trophozoites (containing pigment and a single nucleus), and schizonts (developmental forms with more than one nucleus). Tables 2 and 3 summarize the IC_{50} values for oligodeoxynucleotides listed in Table 1 and for chloroquine versus the chloroquine-resistant (W2) and the chloroquine-sensitive (D6) strains. The parasite cultures were synchronized by D-sorbitol treatment (24), and screenings were performed by incubations with the tested compound for 24 hr (*i*) starting 24 hr after synchronization for the effects on the schizont-to-ring transition or (*ii*) starting 48 hr after synchronization for the effects of compounds on ring-to-trophozoite-to-schizont transitions. At the 72-hr point after synchronization, incubation of the cultures with [^{3}H]hypoxanthine (25) (5 μCi per well; 1 μCi = 37 kBq) afforded incorporation of the radioactive label into the parasite's nucleic acids. Incubations were performed for 4 hr in supplemented media without plasma and without unlabeled hypoxanthine. Incorporation of [^{3}H]hypoxanthine for 24 hr in complete supplemented medium (26) from 48 to 72 hr after synchronization yielded results similar to those obtained for 4 hr (at 72–76 hr after synchronization) with regard to the antimalarial activities of the various compounds that were tested. Comparable results were obtained by the use of nonsynchronous cultures of *P. falciparum* strains and by counting intraerythrocytic forms of the parasite instead of quantification by [^{3}H]hypoxanthine incorporation.

RESULTS AND DISCUSSION

Experiments using ^{32}P-labeled or ^{3}H-labeled oligodeoxynucleotides demonstrated that human erythrocytes are impermeable to this class of compounds (unpublished data), a finding that was reported earlier for rabbit erythrocytes (27). However, the *P. falciparum*-infected erythrocyte was found by autoradiography followed by staining to allow the entry of ^{32}P-labeled or ^{3}H-labeled oligodeoxynucleotides—a crucial difference from the uninfected erythrocyte (unpublished data). Thus, the cell infected by a malarial parasite permits transport of oligodeoxynucleotides across the critical membranes, whereas the uninfected erythrocyte does not, a fortunate situation for potential antisense chemotherapy. It also has been reported (28) that serum macromolecules are

Table 2. Antimalarial activities of oligodeoxynucleotides against the synchronized chloroquine-resistant *P. falciparum* W2 strain

Oligomer or chloroquine (during schizont-to-ring transition, 24–48 hr after synchronization)	IC_{50},* μM	Oligomer or chloroquine (during ring-to-trophozoite-to-schizont transition, 48–72 hr after synchronization)	IC_{50},* μM
PSI	0.9	PSI	>2.5
PSII	1.1	PSII	>2.5
PSIII	0.7	PSIII	>2.5
PSNIII	0.5	PSNIII	>2.5
RI	0.5	RI	>2.5
RII	>5.0	RII	>5.0
RIII	>5.0	RIII	>5.0
Chloroquine†	0.065	Chloroquine†	0.050

*IC_{50} values are based on a 4-hr [^{3}H]hypoxanthine incorporation 72–76 hr after synchronization.

†It should be noted that although the IC_{50} of chloroquine is low in terms of μM concentrations, its toxicity towards the host is significant at these levels of blood plasma concentrations, thus allowing for only a narrow therapeutic window. In contrast, studies in mice and rats (23) indicate toxic effects only at levels of oligodeoxynucleotide phosphorothioates that are at least 20 times the effective IC_{50} levels obtained for these compounds against chloroquine-resistant malaria.

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Table 3. Antimalarial activities of oligodeoxynucleotides against the synchronized chloroquine-sensitive *P. falciparum* D6 strain

Oligomer or chloroquine (during schizont-to-ring transition, 24–48 hr after synchronization)	IC_{50},* μM	Oligomer or chloroquine (during ring-to-trophozoite-to-schizont transition, 48–72 hr after synchronization)	IC_{50},* μM
PSI	0.9	PSI	>2.5
PSII	0.9	PSII	>2.5
PSIII	0.8	PSIII	>2.5
PSNIII	0.5	PSNIII	>2.5
RI	0.7	RI	>2.5
RII	>5.0	RII	>5.0
RIII	>5.0	RIII	>5.0
Chloroquine*	0.015	Chloroquine†	0.004

*IC_{50} values are based on a 4-hr [^{3}H]hypoxanthine incorporation 72–76 hr after synchronization.

†Note that the IC_{50} for chloroquine in this chloroquine-sensitive strain is 6–12 times as low as in the chloroquine-resistant strain (Table 2).

able to gain direct access to intraerythrocytic malarial parasites through a parasitophorous duct.

The data in Tables 2 and 3 indicate that oligodeoxynucleotide phosphorothioates are effective in inhibiting the growth and invasion of chloroquine-resistant and chloroquine-sensitive clones of *P. falciparum*. Analysis of the developmental stages most susceptible to inhibition by these oligomers suggests that the oligomers interfere with one of the following events: schizont maturation, merozoite release, merozoite attachment to red blood cells, merozoite invasion, or ring formation. All of the oligodeoxynucleotide phosphorothioates having a complementary sequence ("antisense") to segments of the malarial genome possess varying degrees of antimalarial activities (PSI, PSII, PSIII, and PSNIII), whereas among the random or seemingly random sequences, only one such sequence, RI, demonstrated comparably significant antimalarial activity. This particular oligomer (RI), however, possesses a 3′-terminal sequence 5′-CAT-3′ which is complementary to the translation start codon 5′-AUG-3′. It can be deduced from Tables 2 and 3 that the oligodeoxynucleotide phosphorothioates exhibit similar antimalarial activities towards chloroquine-resistant and chloroquine-sensitive strains of *P. falciparum* and that these compounds act against a stage in the intraerythrocytic life cycle of the parasite that is closer to schizont maturation and merozoite invasion than to the target stage of chloroquine, a known schizontocidal agent. It is also important to note that the activity of the oligomers is dependent on their resistance to degradation catalyzed by nucleases, since they were added only once every 24 hr. In this regard, the higher antimalarial activity of PSNIII as compared with PSIII is attributed to the butyl phosphoramidate group at the last internucleotide phosphate moiety of the 3′ end, which is the only structural chemical difference between PSNIII and PSIII. Such a chemical modification is found to inhibit exonucleolytic activities catalyzing the degradation of oligodeoxynucleotide phosphorothioates. The data in Table 4 describe the effects of a 48-hr exposure of nonsynchronous W2 strain in culture to a variety of oligonucleotides. The developmental form of the parasite most affected by oligodeoxynucleotide phosphorothioates was the ring form, an observation consistent with the data obtained from synchronous cultures (Tables 2 and 3). Other considerations with regard to the pronounced antiplasmodial activity of the oligomer RI with a seemingly random sequence are that it has been found (29) that as little as two to four complementary bases in a DNA·RNA hybrid are sufficient to activate RNase H, a ubiquitous cellular enzyme (30). The catalytic activity of RNase H hydrolyzes the RNA

Table 4. Antimalarial activity of oligodeoxynucleotides against nonsynchronous chloroquine-resistant *P. falciparum* W2 strain

Oligomer	Conc., μM	Parasitemia* (% parasitized erythrocytes)			[^{3}H]Hyp incorporation, % of control
		R	T	S	
Exp. 1					
None	—	1.8	2.8	2.0	100
PSI	0.1	0.8	2.6	1.4	121
PSI	1	0.1	0.5	0.3	31
PSII	0.1	0.9	1.9	1.3	110
PSII	1	—	0.4	0.3	36
POII	1	0.5	1.5	1.7	88
PNII	1	0.7	1.6	1.8	93
RI	0.1	0.7	1.3	1.5	71
RI	1	0.1	—	0.1	18
RII	0.1	1.1	2.3	2.1	115
RII	1	0.6	1.5	1.0	73
Exp. 2					
None		2.1	1.9	2.3	100
PSIII	0.1	0.9	1.7	2.0	85
PSIII	1	0.2	0.6	0.1	36
PSNIII	0.1	0.7	1.1	0.9	76
PSNIII	1	—	0.3	0.2	20

Nonsynchronous cultures were treated with oligomers for a total of 48 hr with one change of medium and oligomers at 24 hr. [^{3}H]Hypoxanthine ([^{3}H]Hyp) incorporation was performed for 4 hr after the 48-hr treatment period. Conc., concentration.

*Parasitemia was determined by counting a total of at least 1000 erythrocytes. R, T, and S represent the count of rings, trophozoites, and schizonts, respectively.

strand of the DNA·RNA hybrid, thus contributing to translation inhibition. In a targeted genome whose complete sequence is not known, it is therefore difficult to call a defined sequence "random," and the term mismatch seems preferable.

The use of oligodeoxynucleotides (phosphodiester) complementary to sequences of *P. falciparum* DHFR-TS mRNA for the inhibition of cell-free protein synthesis has been described (31). Inhibition of cell-free translation of DHFR-TS mRNA required long oligomers, a preannealing temperature of 65°C (unacceptable in *in vivo* studies) and resulted in significantly higher IC_{50} values than micromolar levels (31). In this regard it is important to note that unmodified oligomers such as POII or its phosphomorpholidate derivative PNII were much less inhibitory than the corresponding oligodeoxynucleotide phosphorothioate PSII in *P. falciparum* in culture (Table 4). Oligodeoxynucleotide phosphorothioates possess more of a polyanionic character than do oligodeoxynucleotides or their uncharged phosphomorpholidates. Polyanions, including high molecular weight compounds such as heparin or dextran sulfate, have been shown to inhibit invasion of erythrocytes by *P. falciparum* by mechanisms that presumably affect the interaction between merozoites and sialic acid on the erythrocyte membrane (32). The polyanionic character of oligodeoxynucleotide phosphorothioates would suggest an additional explanation for a non-sequence-specific component in the inhibition of *P. falciparum* by these compounds, as exemplified by the activity of oligomers such as RI. We should note, however, that an oligodeoxynucleotide phosphorothioate complementary in sequence to nucleotides 16–36 of the open reading frame from the start codon of *P. falciparum* DHFR-TS gene (14) yields antiplasmodial activities that are almost identical to those obtained with PSIII. The oligodeoxynucleotide phosphorothioate complementary in sequence to nucleotides 16–37 (a 22-mer) of the same gene produced distinctly better antiplasmodial effects than either one of the 21-nucleotide-

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long oligomers (data not shown). The sequence-dependent effects of these oligonucleotides are in most likelihood the major component of the antimalarial activities of this class of compounds.

In summary, oligodeoxynucleotide phosphorothioates and their chemical modifications were shown to be effective in inhibiting growth and invasion of drug-resistant as well as drug-sensitive *P. falciparum* in culture. Thus, it is suggested that oligonucleotide drugs possess structural features that enable them to bypass cross-resistance to multiple drugs. This cross-resistance has in large part been responsible for emergence of the recent global spread of malaria (1).

We thank Dr. Craig Wilson of the Harvard School for Public Health for his advice and technical instruction. This work was supported by grants from the G. Harold and Leila Y. Mathers Foundation, and National Institutes of Health Grants AI-24846 and P30-12708-17.

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