Inhibition of \textit{Plasmodium falciparum} Proliferation \textit{in Vitro} by Ribozymes*

(Received for publication, January 14, 1997, and in revised form, May 8, 1997)

Maria Vega C. Flores§*, David Atkins*, Denis Wade*, William J. O'Sullivan*, and Thomas S. Stewart‡

From the §School of Biochemistry and Molecular Genetics, University of New South Wales, Sydney 2052, Australia and ¶Johnson & Johnson Research Proprietary Limited, 154 Pacific Highway, St. Leonards, New South Wales 2065, Australia

Catalytic RNA (ribozymes) suppressed the growth of the human malarial parasite \textit{Plasmodium falciparum \textit{in vitro}}. The phosphorothioated hammerhead ribozymes targeted unique regions of the \textit{P. falciparum} carbamoyl-phosphate synthetase II gene. The \textit{P. falciparum} carbamoyl-phosphate synthetase II gene encodes the first and limiting enzyme in the pathway, and its mRNA transcript contains two large insert regions absent in other carbamoyl-phosphate synthetases, including that from humans. These inserts are ideal targets for nucleic acid therapy. Exogenous delivery of ribozymes to cultures reduced malarial viability up to 55% at 0.5 \(\mu\text{M}\) ribozyme concentrations, which is significantly greater than control levels (5–15% reduction), suggesting a sequence-specific inhibition. This inhibition was shown to be stage-specific, with optimal inhibitions being detected after 24 h, coincident with maximal production of the carbamoyl-phosphate synthetase enzyme in the course of the life cycle of the parasite. A decrease in total carbamoyl-phosphate synthetase activity was observed only in cultures treated with the ribozymes. The task of developing alternative therapeutic agents against malaria is urgent due to the evolution of drug-resistant strains of \textit{P. falciparum}, the most virulent of all human malarial parasites. Another critical issue to be addressed is the possibility of eliminating or reducing any systemic toxicity to the host, which can potentially be provided by nucleic acid therapy. This work is the first reported assessment of the ability of ribozymes as antimalarials. Ribozyme inhibition assays can also aid in identifying important antimalarial loci for chemotherapy. The malarial parasite can, in turn, be a useful \textit{in vivo} host to study the catalysis and function of new ribozyme designs.

Malaria remains a major health problem not only in Africa and Asia, where it afflicts >200 million people annually, but also for migrants and travelers due to unreliable drug regimes (1). The important factors that preclude the eradication of malaria are the emergence of parasite and vector resistance to drugs and insecticides and the slow development of universal strategies for malaria vector control (2). New antimalarials and limiting enzyme in the pathway, and its mRNA transcription contains two large insert regions absent in other carbamoyl-phosphate synthetases, including that from humans. These inserts are ideal targets for nucleic acid therapy. Exogenous delivery of ribozymes to cultures reduced malarial viability up to 55% at 0.5 \(\mu\text{M}\) ribozyme concentrations, which is significantly greater than control levels (5–15% reduction), suggesting a sequence-specific inhibition. This inhibition was shown to be stage-specific, with optimal inhibitions being detected after 24 h, coincident with maximal production of the carbamoyl-phosphate synthetase enzyme in the course of the life cycle of the parasite. A decrease in total carbamoyl-phosphate synthetase activity was observed only in cultures treated with the ribozymes. The task of developing alternative therapeutic agents against malaria is urgent due to the evolution of drug-resistant strains of \textit{P. falciparum}, the most virulent of all human malarial parasites. Another critical issue to be addressed is the possibility of eliminating or reducing any systemic toxicity to the host, which can potentially be provided by nucleic acid therapy. This work is the first reported assessment of the ability of ribozymes as antimalarials. Ribozyme inhibition assays can also aid in identifying important antimalarial loci for chemotherapy. The malarial parasite can, in turn, be a useful \textit{in vivo} host to study the catalysis and function of new ribozyme designs.

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* This work was supported by Johnson & Johnson Research Pty. Ltd. and by Australian Research Council Collaborative Grant G295301074. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Fax: 61-2-93851483; E-mail: m.flores@unsw.edu.au.

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1 L. Grover, W. J. O'Sullivan, and T. S. Stewart, manuscript in preparation.

2 The abbreviation used is: pfCPSII, \textit{P. falciparum} carbamoyl-phosphate synthetase II.
Antimalarial Ribozymes

Mammalian Carbamoyl Phosphate Synthetase

**Fig. 1. Location of cleavage sites of CPSRz1 and CPSRz4 in the pfcPSII gene.** The diagram compares the primary structure of the carbamoyl-phosphate synthetase II gene in mammals and *P. falciparum*, emphasizing the insertion of large sequences positioned between junctions of the defined carbamoyl-phosphate synthetase (CPS) domains, the glutamine aminotransferase (GAT) and synthetase domains. The mammalian gene is part of a multifunctional complex also encoding aspartate transcarbamylase (ATCase) and dihydroorotase (DHOase) (12). Arrows denote the cleavage sites of CPSRz1 and CPSRz4. *3′* of the consensus GUC ribozyme cleavage motif (18). *kb*, kilobases; *a.a.*, amino acids.

Ribozymes can be delivered to cells either endogenously or exogenously. Intracellular production of ribozymes is achieved by transcription from a transient or an integrated gene, whereas synthetic ribozymes are administered like conventional drugs. The main advantage of using synthetic ribozymes is the possibility of developing more nuclease-resistant oligonucleotides. However, the uptake inefficiency of these large molecules (8) must also be taken into account.

This work tests the feasibility of exogenous delivery of synthetic ribozymes as antimalarials and the potential of the pfCPSII gene as a target for such therapy. Targeting the insert regions of the gene could provide confirmation that these unique regions are integral in the pfCPSII mRNA. We show that simple ribozymes can inhibit growth of *P. falciparum* in a sequence-dependent manner. Furthermore, using an effective antisense deoxyoligonucleotide directed against the dihydrofolate reductase gene (DHFR105) (17), we demonstrate that the carbamoyl-phosphate synthetase ribozymes can perform as well as and potentially better than DHFR105.

**EXPERIMENTAL PROCEDURES**

**Malaria Cultures**—The FCQ-27/Papua New Guinea (chloroquine-sensitive) and K1/Thailand (chloroquine-resistant) strains of *P. falciparum* were grown in continuous culture in type O serum. All cultures were maintained in a modified version of the techniques described by Trager and Jensen (20) as previously reported (21). The parasite strains were provided by the Army Malaria Research Unit (Ingleburn, Australia). Blood and serum were obtained from the Blood Bank of Australia. Human serum was pooled from a minimum of 10 donors, aliquoted, and frozen until use.

**Oligonucleotide Synthesis**—All oligonucleotides were prepared by Dr. P. Hendry (Division of Biomolecular Engineering, Commonwealth Scientific and Industrial Research Organization, Sydney, Australia) using methods described earlier (22).

**In Vitro Ribozyme Cleavage**—200 ng of BglII-linearized pTZ18U containing a portion of the pfCPSII gene encoding the first insert region and an EcoRI-linearized pTZ19U containing sequence for the second insert were transcribed in vitro with T7 RNA polymerase (Promega) and 240 pmol of α-32P-UTP (Amersham Corp.) using methods previously described (23). The cleavage conditions were essentially as described (23), using 20 pmol of RNA substrate and 100 pmol of ribozymes in a total volume of 50 μl. Aliquots (5 μl) were taken at set time points and mixed with stop solution (100% formamide, bromphenol blue, and xylene cyanol). Sources were heated to 90 °C for 5 min and resolved by polyacrylamide gel electrophoresis. After vacuum-drying the gel, the cleavage products were visualized and quantified using a PhosphoImager xylene cyanol). The samples were heated to 90 °C for 5 min and resolved by polyacrylamide gel electrophoresis. After vacuum-drying the gel, the cleavage products were visualized and quantified using a PhosphoImager.

**Malaria Inhibition Assays**—The assay previously used to establish the antimalarial activity of DHFR105 (17) was adopted for this study. Parasite growth data were determined either by microscopy alone or using the “corrected value” (parasite cell count multiplied by [H]hydroxyoxazine incorporation)/17. Experiments were performed blind, and the “corrected value” was revealed following analysis. A variation of the above assay for synchronous ring-stage malaria (25) was carried out after exposure of cells to oligonucleotides for 24 or 48 h. All values presented are the average of three experiments of triplicate samples for each oligonucleotide dilution. Differences in growth reduction compared with controls were analyzed by Student's *t* test using GraphPAD Prism (GraphPAD Software for Science).

**Enzyme Assays**—A microassay for carbamoyl-phosphate synthetase activity was performed on crude lysates from 500-μl cultures. The colorimetric assay for lactate dehydrogenase was performed as described (27). Enzyme activities were determined for triplicate samples from three malaria inhibition assays. The parasites were synchronous and were incubated with or without 0.5 μM oligonucleotides for 24 h.

**RESULTS**

CPSRz1 and CPSRz4 are all-RNA trans-acting hammerhead ribozymes (19) with 14-base hybridizing arms. 10 bases at both the 5′- and 3′-ends are phosphorothioated in an attempt to render nuclease stability. They were designed to cleave mRNA within the unique insert regions in the pfCPSII gene (5) absent in all carbamoyl-phosphate synthetase genes, including that from mammals (12) (Fig. 1). CPSRz1 targets nucleotides 894–921 (insert 1), and CPSRz4 targets nucleotides 3719–3748 (insert 2). A catalytically inactive control ribozyme (RzM) bears the exact sequence of CPSRz4 except for two mutations, G5U and A14G (underlined in Table I), in the catalytic domain (28). An antisense control was prepared that contains the complementary arms of CPSRz4 as a fully phosphorothioated RNA molecule (As4). The controls were designed to determine if inhibition by CPSRz4 was via substrate mRNA cleavage rather than antisense-dependent mechanisms. Two controls (RzN and RzX, ribozymes bearing nonhybridizing arms) were prepared to determine the level of sequence-independent inhibition of molecules containing phosphorothioates. These controls have randomized arm sequence, but maintain an active catalytic domain. A well characterized antisense deoxyoligonucleotide, DHFR105 (17), targeting the dihydrofolate reductase gene was used as a reference control for this study. Unlike the former oligonucleotides, DHFR105 is a fully phosphorothioated DNA molecule, a conventional design in antisense technology.

**In vitro cleavage analysis of CPSRz1 and CPSRz4** showed that both ribozymes cleaved the radiolabeled pfCPSII mRNA fragment. Analysis of the time course of *in vitro* cleavage of CPSRz4 (Fig. 2) shows that it has a cleavage rate (*t*1/2) of 8 min and is comparable with other studies of ribozymes with similar arm lengths (23). Under the same conditions, no *in vitro* cleavage of the pfCPSII transcript was observed with RzM, RzN, and RzX (data not shown).

To test that the ribozymes were not toxic to the host, cell doubling times as well as nucleic acid synthesis levels of several mammalian cell lines (DAMI, K562, and MOLT4) were shown to be unaffected by all test conditions, which extended to...
time points were analyzed on a 7.0 M urea, 6% polyacrylamide gel. The enzymed medium was thus used for all assays. Serum-supplemented medium was supplemented with 10% human serum (pooled from a minimum of 10 donors, not heat-inactivated), the ribozyme concentrations above 2 μM, with CPSRz4 being marginally more effective than CPSRz1 (data not shown). When the medium was supplemented with 10% human serum (pooled from a minimum of 10 donors, not heat-inactivated), the ribozymes maintained their antimalarial action. Serum-supplemented medium was thus used for all assays.

Similar to the findings of Barker et al. (17), a stimulation of [3H]hypoxanthine incorporation (cpm) was detected in the cultures upon incubation with the various oligonucleotides. In this study, no considerable decrease in cpm was observed at oligonucleotide concentrations below 1.0 μM. This meant that quantification of growth reduction by measuring [3H]hypoxanthine uptake was redundant. Therefore, assessments of further experiments were performed counting viable parasite cells by microscopic examination.

Asynchronous cultures harvested after a 48-h incubation with ribozymes were significantly inhibited by DHFR105 (positive control) and CPSRz4 compared with the other control ribozymes (p < 0.001) (Fig. 3A). Whereas RzM, RzN, and As4 produced very minimal reduction of parasite growth (10–15%) throughout the range of oligonucleotide concentrations, CPSRz4 showed increasing suppression starting from 100 nM concentrations, almost as effective as the dihydrofolate reductase antisense deoxyoligonucleotide (DHFR105). Inhibition by DHFR105 reached 60% and that by CPSRz4 reached 40% at 0.5 μM oligonucleotide concentrations.

To further compare the efficacy of CPSRz4 and DHFR105, 48-h assays were performed using synchronized parasites at ring stage at the start of the experiment (t = 0). In this assay, the parasites proceed through their full life cycle, i.e., from ring forms to the next generation of daughter rings. The pattern of inhibition was less overall than asynchronous assays with the subset of oligonucleotides tested (Fig. 3B). CPSRz4 gave a total inhibition of only 30%. Interestingly, DHFR105 inhibition also dropped to a similar level, a decrease in effectiveness of almost half compared with results from asynchronous cultures (Fig. 3A). The percent reduction by CPSRz4 and DHFR105 was maintained at 6-fold greater levels compared with controls.

We also examined the levels of growth suppression of synchronous cultures after a 24-h exposure to a subset of oligonucleotides at a range of concentrations below 0.5 μM. The parasites were grown from ring forms to mature trophozoites, when they occupy almost the whole of the erythrocytic cytoplasm. This was devised to monitor stage-specific inhibition by CPSRz4. Inhibition by CPSRz4 was observed at concentrations as low as 20 nM, which was not seen in controls including DHFR105. This effect peaked at 55% suppression at 0.5 μM, whereas DHFR105 reached 30% inhibition (Fig. 3C).

An initial indication whether inhibition by ribozymes was reflected in decreased carbamoyl-phosphate synthetase production was sought. Crude protein extracts from parasites incubated with 0.5 μM oligonucleotides for 24 h were prepared to determine the levels of carbamoyl-phosphate synthetase activity. P. falciparum lactate dehydrogenase activity (27) was also determined for comparison. Whereas no significant drop in lactate dehydrogenase activity was observed in parasites treated with the different oligonucleotides compared with untreated cultures (data not shown), a notable reduction in carbamoyl-phosphate synthetase activity was detected in parasites incubated with CPSRz4 (Fig. 4). A decrease of almost 50% of the induced levels of carbamoyl-phosphate synthetase activity over basal levels was observed.
DISCUSSION

The revolutionary move to develop nucleic acid therapy in clinical medicine paved by studies in cancer and AIDS (8) is advancing in parasitology. Recent success in delineating a sequence-specific response to antisense therapy in *P. falciparum* in a range of gene targets (17) shows the potential of this technology.

This work investigated the susceptibility of *P. falciparum* to ribozyme action via the pfCPSII mRNA target. This is the first report of administering ribozymes to malarial parasites to elicit growth inhibition. The application of this technology to the malarial system favors the use of chemically synthesized ribozymes over endogenous delivery for several reasons. The mature erythrocytes are apparently impermeable to oligonucleotides (30), but uptake is markedly increased once the cells are infected with malaria (11). Whereas oligonucleotides of up to 20 bases can enter cells by distinct receptor-mediated mechanisms, larger and more charged molecules, like ribozymes, seem unlikely to be internalized in the same way (31). However, recent reports suggest that uptake of nucleic acids may vary depending on cell type (32–34).

The task of getting nucleic acids into malaria is even more complicated than in mammalian cells due to the presence of several membrane layers: the red blood cell membrane, the parasitophorous vacuole, and the parasite's cell membrane. Stable transfection of *P. falciparum* by plasmids was achievable by electroporation, but cannot be fully attributed to it (35). It has been suggested that the possibility of an easier access to the intraerythrocytic space surrounding the parasite via parasitophorous ducts (36) may clarify this phenomenon. This could

**Fig. 3.** Effect of varying concentrations of oligonucleotides on the growth of *P. falciparum* in culture. Oligonucleotides (0–0.5 μM) were administered in triplicate parasite cultures at *t* = 0 under the following assay conditions: asynchronized parasites, 0.5% starting parasitemia, and 48-h incubation (A); synchronized parasites, 0.5% starting parasitemia, and 48-h incubation (B); and synchronized parasites, 2.0% starting parasitemia, and 24-h incubation (C). Parasite cell count was determined after the incubation period, and percent growth was calculated from average parasitemia in untreated controls. The differences from 100% (control average) are expressed as percent reduction on the ordinate. The data are means ± SD of three experiments, each having triplicate cultures. DHFRAs, dihydrofolate reductase antisense deoxyoligonucleotides.
S.D. DHFRAs indicates induced enzyme activity after 24 h; and the mRNA to be inactivated in the course of the experiment. Since Presuming that the gene targets are expressed stage-specifically, different life forms of the asexual parasite are present.

CPSRz4 in reducing parasite growth by almost 8-fold due to its greater stability and the complementary action of RNase H. Although both As4 and RzM hybridize to pfCPSII owing to its RNA phosphorothioate composition, mimicking the complementary arms of CPSRz4. A DNA phosphorothioate analogue, commonly used in therapeutic antisense design, would be expected to have greater inhibitory properties due to its greater stability and the complementary action of RNase H. Although both As4 and RzM hybridize to pfCPSII mRNA, only RNA-RNA interactions are formed. Inactivation is therefore limited to direct blockade of the translation machinery since RNase H-mediated inhibition requires RNA-DNA hybrids. The low levels of growth reduction afforded by As4 and RzM do not differ significantly, so it can be inferred that they inactivate via the same mechanism. Hence, the observed efficiency of CPSRz4 in reducing parasite growth by almost 5-fold (Fig. 3A) is due to inactivation by catalytic cleavage.

When synchronous, instead of asynchronous, cultures were used in a 48-h assay (Fig. 3B), similar levels of inhibition (30%) were observed for CPSRz4 and DHFR105. This meant that the percent reduction exerted by DHFR105 decreased by half. The effect of CPSRz4 also went from 40 to 30%. In an asynchronous situation, different life forms of the asexual parasite are present and are developing through the cycle in a 48-h period. Presuming that the gene targets are expressed stage-specifically, there will consequently be a constant level of target mRNA to be inactivated in the course of the experiment. Since DHFR105 is far more stable than CPSRz4 in a 48-h period (data not shown), one expects the antisense to perform better, as we have observed (Fig. 3A). However, in a synchronous culture, when only one population of the parasite is subjected to inhibition, the levels of growth suppression for both oligonucleotides have consistently been similar (Fig. 3B). This suggests that both antagonists perform equally well against their respective loci within a certain time frame in the 48-h life cycle of malaria. Theoretically, these time frames have to coincide with optimal levels of gene expression and enzyme production of the target genes. Previous studies have shown maximal production of the pyrimidine de novo enzymes at ~24–27 h of the parasite’s life cycle (4). There is also evidence that dihydrofolate reductase gene expression in P. falciparum peaks after 36 h (11).

Monitoring CPSRz4 inhibition of synchronized parasites for 24 h (i.e., from ring form to trophozoite stage) demonstrated a reduction in malarial viability at concentrations as low as 20 nM (Fig. 3C). Maximal suppression at 0.5 μM was 3–5-fold greater than with the control constructs (RzM and As4). Using this assay, CPSRz4 performed 2-fold better than DHFR105. This clearly shows that the time of inhibition by CPSRz4 precedes that by DHFR105, and in an experimental window of 24 h, during the maturation of rings to trophozoites, CPSRz4 is a better inhibitor.

The inhibitory effect of a single dose of CPSRz4 linearly increased over a 24-h time course (data not shown). This may reflect two things: first, an increased efficiency of the parasites to take up oligonucleotides as they develop; and second, an increased availability of target molecules in preparation for schizogony. One or both of these factors may be at play, but this will need further clarification. The current data indicate a time-dependent inhibition by CPSRz4 and DHFR105, the former taking effect at least 12 h earlier than the latter. If onset of parasite death is conditioned by factors other than mRNA inactivation, the action of DHFR105 should have taken effect at 24 h or even earlier, as cells are, in theory, more permeable to DNA. DNA is also more stable than RNA. This, however, was not the case, pointing to a stage-specific inhibition. The sequence- and stage-specific suppression by CPSRz4 was evident in the concomitant decrease of carbamoyl-phosphate synthetase activity recovered only from cultures incubated with CPSRz4 (Fig. 4). Although DHFR105 exerted a 30% inhibition of parasite growth, no significant decrease in carbamoyl-phosphate synthetase activity was detected in these cells. This correlates with the reported maximal production of carbamoyl-phosphate synthetase after 24 h. Furthermore, there were no measurable changes in enzyme levels for the glycolytic enzyme, lactate dehydrogenase, in all test cultures (data not shown).

The efficacy of DHFR105 in our study was less than the published data (17), reaching >80% at 0.5 μM. However, the overall background levels of suppression for control oligonucleotides were also consistently lower in our experiments, the highest being 15% at 0.5 μM compared with 40% from the previous study. The discrepancy may be simply due to variations in the synthesis and purification protocols of the antisense used.

The data presented clearly demonstrate the susceptibility of the pfCPSII mRNA as a target for ribozymes. For this study, the ribozymes were directed against the large unique insert sequences. The pfCPSII gene provides one of the most dramatic examples of these structures, making up 35% of its total sequence. Although found in a representative of P. falciparum genes, the functions of these regions remain as speculations, the most popular being their involvement in the control of stage- or species-specific gene transcription during the developmentally complex life cycle of the parasite (37). The accessibility of the insert regions of pfCPSII to ribozyme cleavage...
confirms previous data that they remain intact in the functional mRNA (5). The ribozyme assay in malaria can be a powerful tool to confirm structural and functional characteristics of genes. More important, it can be very useful to test and identify relevant loci for antimalarials before embarking on long-term chemotherapeutic projects.

The benefits of nucleic acid therapy in treating acute malaria, although promising, are dependent on improved technology and lower production costs for clinical application. Future directions on our part include improvement of ribozyme stability and uptake. Effective inactivation of a gene is conditioned by the accessibility of the sequence targeted, as seen studies with the dihydrofolate reductase gene (11, 17). This may be directed by the mRNA secondary structure in vivo. A more detailed survey of other loci within the pCPSII gene will be conducted to see any improvements in the current level of inhibition afforded by CPSRz4.

Another more achievable application arising from this investigation is the suitability of P. falciparum as an in vivo model to conduct comprehensive analysis of the multiple parameters of ribozyme function. There are also current advances in ribozyme designs for nuclease resistance and increased catalysis (26, 38–40) that equally require a experimental host for thorough assessment.

The issue of exogenous ribozyme delivery is not an apparent problem in the malarial system due apparently to differential uptake by malaria-infected erythrocytes (11). Thus, apart from sequence-dependent inhibition, possible host cellular toxicity is lowered because the ribozymes are naturally targeted to red blood cells bearing parasites. This has more advantage over the common exogenous delivery of oligonucleotides by liposome encapsulation, which does not differentiate between cell types. The use of cationic lipids for malaria is not applicable because of the apparent prerequisite for a serum-free environment and their relative toxicity to cells (7). There are additional benefits in using the P. falciparum cultures: (i) assays can be performed in as little as 100 μl of culture, thus lowering ribozyme production costs; and (ii) since the culture medium is supplemented with 10% human serum, and not bovine serum, which is used with other mammalian cell lines, it serves as a better model for clinical application.

We have demonstrated the potential of ribozymes as antimalarials and as genetic tools to screen for essential chemotherapeutic targets. The malarial system can also be very useful to test in vivo the performance of improved designs of hammerhead ribozymes.

Acknowledgments—We thank M. Katrib and S. M. B. Eisele for excellent technical assistance and Prof. W. Gerlach for interest in and valuable suggestions for this project.

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