Communications:

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Inhibition of *Plasmodium falciparum* Protein Synthesis

TARGETING THE PLASTID-LIKE ORGANELLE WITH THIOSTREPTON*

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The human malaria parasite *Plasmodium falciparum* has two extrachromosomal DNAs associated with organelles whose function is unclear. Both genomes encode ribosomal RNAs (rRNAs) that are distinct from the nuclear-encoded rRNAs. Secondary structure analysis of all the *P. falciparum* rRNAs indicates that only the large subunit (LSU) rRNA encoded by the plastid-like genome is the target for thiostrepton. Indeed we find that thiostrepton inhibits growth of the parasite in the micromolar range which is 10-fold below concentrations with observable effects on total protein synthesis. We have further examined selective effects of thiostrepton on the plastid function by comparing differential effects of the drug on cytoplasmic and organellar encoded transcripts. Treatment with either thiostrepton or rifampin, an inhibitor of organellar and eubacterial RNA polymerase, both showed disappearance of organellar-encoded RNA transcripts within 6 h of treatment while transcripts of a nuclear-encoded mRNA remained constant for at least 8 h of treatment. Hence, we show a selective effect on organelle function that is suggestive of interference in the protein synthesis apparatus of the plastid. Sensitivity of *P. falciparum* to thiostrepton confirms that the plastid-like genome is essential for the erythrocytic cycle and presents a novel therapeutic site for this class of antibiotics.

Plasmodium, the agent responsible for malaria, is an obligate intracellular parasite that contains three genomes, all of which encode rRNAs. Aside from the nuclear genome, these parasites have two extrachromosomal DNAs, a 6-kb[1] linear genome and a 35-kb circular genome. The smaller extrachromosomal genome is believed to be mitochondrial and encodes fragmented rRNAs and mitochondrial proteins (cytochrome oxidase I and cytochrome *b*) (1). The larger extrachromosomal genome encodes a complete set of rRNAs as a palindromic repeat, tRNAs, ribosomal proteins, and proteins including homologs of subunits of eubacterial RNA polymerase (2, 3). This extrachromosomal element is believed to be of plastid origin, based on sequence comparison and the arrangement of the rRNA genes. The mitochondrion has been visualized (4), and the plastid-like organelle has been localized in *Plasmodium falciparum* (5). Both organelles have also been localized and are distinct in the related Apicomplexan *Toxoplasma gondii* (6). The presence of the plastid-like genome raises the question as to its origin and function in the evolved intracellular parasite (5). Selective inhibition of the organelle would lead to an understanding of its function, as the organellar transcripts vary in abundance during the erythrocytic cycle (7).

Many antibiotics bind catalytic sites in the rRNA and inhibit protein synthesis (8). The availability of sequences of the complete set of the stage-specific, developmentally regulated rRNA genes of *Plasmodium* allows an investigation of predicted sites for interaction of drugs between the organellar- and nuclear-encoded rRNAs (1, 9–11). The thiazole-containing peptide antibiotics, of which thiostrepton is an example, inhibit translation and ribosomal GTPase activity by binding to a limited and conserved region in the large subunit (LSU) rRNA found in eubacteria and organelles and not the corresponding region in eucarya (12–14). This region is referred to as the GTPase domain and also binds ribosomal protein L11 (L12 in eucarya) (12, 15). The structure of the GTPase domain is conserved between eucarya and eubacteria, with characteristic variation at limited positions for each kingdom (16). Thiostrepton directly interacts with two nucleotides in the GTPase domain of *Escherichia coli*. Base substitution or methylation at one of these positions (nucleotide A^{1067}) causes thiostrepton resistance (17). In contrast, universal protein synthesis inhibitors, such as anisomycin, bind to sites conserved in all rRNAs (8).

In this study, we test the ability of thiostrepton to specifically inhibit the plastid-like organellar protein synthesis, as compared with a universal protein synthesis inhibitor, and investigate the effect of the inhibition of this organelle on growth of the parasite.

MATERIALS AND METHODS

Assay of Growth Inhibition—Thiostrepton (1525 units/mg; Calbiochem) and anisomycin (Sigma) were dissolved at 100 mM in dimethyl sulfoxide (Pierce). *P. falciparum* (strain 3D7) was maintained in culture with human erythrocytes (5% hematocrit) in RPMI 1640 (Life Technologies Inc.) supplemented with HEPES and sodium bicarbonate and human sera (10%) under standard conditions (18, 19). The growth inhibition assay was conducted as described (20). Briefly, the parasitemia was adjusted to 0.1% parasitemia, 2.5% hematocrit, and 200-μl aliquots were placed in wells of a microtiter dish. Serial dilutions of drugs were made in RPMI. Thiostrepton was diluted to 10 mM in dimethyl sulfoxide before the serial dilutions in RPMI. Aliquots (20 μl) were added in triplicate to the cultures in the microtiter plate, mixing well. At the highest concentrations (final 0.2 mM), thiostrepton precipitates. After incubation for 48 h under standard conditions, [2,8-^{3}H]hypoxanthine (Moravek Biochemicals, 12.5 Ci/mmol) in RPMI (20 μl, 0.05 mCi/ml) was added to each well. After incubation for an additional 24 h, the cultures were lysed, and incorporated radioactivity was measured with an automated counter. Each point is an average of three determinations.

Assay of Inhibition of Protein Synthesis—Assay for inhibition of protein synthesis was similar to above except the culture and drug dilutions were in RPMI 1640 without leucine (SelectAmine, Life Tech-

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[1] The abbreviations used are: kb, kilobase(s); LSU, large subunit; PCR, polymerase chain reaction; RT, reverse transcriptase.

nologies, Inc.), and the sera were dialyzed extensively as described (21). Also, the cultures were adjusted to 1% parasitemia prior to the experiment. Microtiter plates were set up with drug dilutions as described above except cultures were incubated with drugs for only 4 h. [3,4,5-^{3}H]Leucine (Moravek Biochemicals, 122 Ci/mmol, 0.5 mCi/ml, 100 μCi) was diluted to 4 ml in RPMI without leucine. The radiolabeled leucine (20 μl, 1 mCi) was added to each well and incubated for another 4 h. The microtiter plates were processed as above to quantitate the incorporated radiolabel.

Assay of Organellar and Cytoplasmic Transcript Levels—RNA polymerase transcript levels were assessed by comparing the amounts of RNA synthesized at time points following drug treatment. Cultures of *P. falciparum* (3.2% parasitemia, 5% hematocrit) were treated with thiostrepton at 8 μM and rifampin (Sigma) at 80 μM, near the IC_{99} values (this study and Ref. 22). Aliquots (5 ml) of treated and control cultures were removed and immediately processed for RNA with a guanidinium thiocyanate solution, according to the manufacturer's directions (RNAgents, Promega). All RNA samples were dissolved in 50 μl of diethyl pyrocarbonate-treated water, DNase I-treated as described previously (23), and an aliquot (1 μl) was removed for reverse transcriptase-PCR. First strand synthesis of cDNA was completed with a random hexamer (SuperScript Preamplification System, Life Technologies, Inc.). One-tenth of the cDNA product was utilized for PCR of *rpoB/C*, MSA1, and rRNA. Primers corresponding to the 3′ region of *rpoB*, 5′-GGGCTTTAGAAGCTTTTGG-3′, and the 5′ region of *rpoC*, 5′-CCATTTAAAATTGGTAATCCTG-3′ were applied as described (2, 3) for PCR of nascent *rpoB/C* transcripts. Reactions were cycled with the following parameters: 94 °C/30 s, 42 °C/30 s, 72 °C/60 s, 35 cycles. Primers for amplification of nucleotides 64 to 614 of MSA1 with 5′-GTGTGATAATATTCATGG-3′ and 5′-GGAGAGCATTTGGTG-3′ (24) and the small subunit rRNA with oligonucleotides 841 and 844 (23) were used for amplification reactions following the parameters in the respective references except with 35 cycles. Samples were also analyzed after 25 cycles of amplification to ensure detection in the linear range of the amplification reaction, with similar results (data not shown). Following electrophoresis of aliquots from the amplification reactions on 1% agarose:TBE gels, samples were transferred to nylon membranes (GeneScreen Plus, DuPont NEN) and hybridized as described (25). The amplification products were probed with 5′-^{32}P-labeled oligonucleotides. The *rpoB/C* products were probed with 5′-GTTTAGCTATTAATATAGAAGC-3′ (nucleotides 2009–2030 of *rpoB*) and 5′-CGGAGAGGTATTAATACC-3′ (nucleotides 108–125 of *rpoC*), in 5 × SSC, 10 mM sodium phosphate, 0.05% sodium pyrophosphate, 1% sodium dodecyl sulfate, 5 × Denhardt's solution, 100 μg/ml yeast tRNA, 42 °C, and washed in the hybridization solution lacking Denhardt's and tRNA at 37 °C, three times. The final wash was 1 × SSC, 0.5% sodium dodecyl sulfate, 42 °C followed by autoradiography. The same results were obtained with either probe. The MSA1 amplification products were similarly probed with 5′-AAACTTGTGTTCGGATATAG-3′ and the rRNA products with oligonucleotide 842 (23).

RESULTS

Rationale—Sequence alignment and analysis of the corresponding secondary structures of the *P. falciparum* nuclear-encoded A- and S-type LSU rRNAs enable comparison with the GTPase domain of these LSU rRNAs with LSU rRNAs encoded on the 35-kb circle and as fragments on the 6-kb linear genome (Fig. 1). The GTPase domain of the nuclear-encoded LSU rRNAs are conserved at most positions shared by other eucaryotes, although the S-type, which is not expressed in blood stages (11), has three differences from the A-type and all other eucaryotes (Fig. 1*A*). The GTPase domain of the 35-kb encoded LSU rRNAs is extremely A + T-rich, although the overall secondary structure resembles typical eubacterial LSU rRNAs (Fig. 1*B*). The previously proposed GTPase domain encoded by the 6-kb linear genome was assembled from two non-contiguous expressed fragments (6B and 6C) (1), which when combined do not complete the secondary structure of a typical GTPase domain (16). Although RNA editing may alter the final transcript, RNA editing has only been observed to date in the trypanosomatid lineage, not in Apicomplexa (the *Plasmodium* lineage). In *P. falciparum*, binding of the ribosomal protein may compensate for the rRNAs encoded as fragments, as the ribosomal protein L11 also binds to this region in *E. coli* (12).

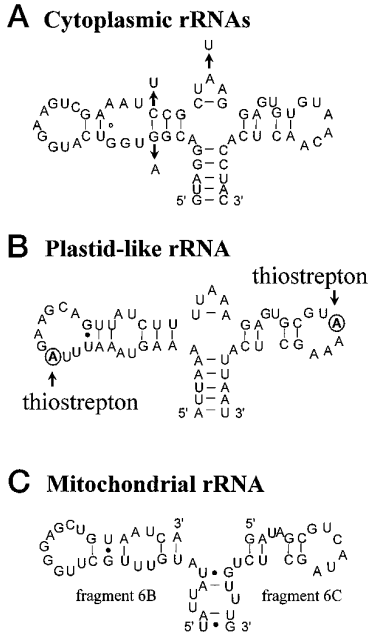

FIG. 1. **The proposed secondary structures of the GTPase domain encoded by large subunit rRNA genes of *P. falciparum*.** *A*, the GTPase site for nuclear-encoded, asexually expressed LSU rRNA gene (A-type), with *arrows* indicating changes in the sporozoite expressed LSU rRNA gene (S-type). The sequences shown correspond to nucleotides 1827–1770 for the A gene, GenBank™ accession number U21939 (10), and nucleotides 2044–2120 for the S gene, GenBank™ accession number U48228 (11). *B*, the GTPase site of the LSU rRNAs encoded by a palindrome within the 35-kb circular DNA. The sequence shown corresponds to nucleotides 1019–1071 for the GenBank™ accession number X61660 (31). Indicated by *arrows* are conserved sites for interaction with thiostrepton, with the 5′-proximal A corresponding to nucleotide 1067 in *E. coli* LSU rRNA that is altered in thiostrepton-resistant mutants. *C*, the proposed GTPase site encoded as fragments by the mitochondrial-like 6-kb linear genome. The sequence shown corresponds to the reverse complement of nucleotides 4624–4587 for fragment 6B and nucleotides 221–200 for fragment 6C from GenBank™ accession number M76611 (44). Secondary structures are drawn according to the model of Ryan *et al.* (13), although an alternative structure for part of the *E. coli* GTPase domain has been proposed (45).

Thiostrepton interacts with both A^{1067} and A^{1095} in *E. coli* LSU rRNA (17). As would be expected for a plastid-like organellar genome of eubacterial origin, the 35-kb circle of *P. falciparum* has A at both of the corresponding positions in the GTPase domain of the LSU rRNA (see Fig. 1). In contrast, the nuclear-encoded and 6-kb fragments encode LSU rRNAs altered at one or both of these positions. Since the primary site of resistance to thiostrepton is either methylation or mutation at the site corresponding to A^{1067} (8), *P. falciparum* should be sensitive to thiostrepton with the target of the 35-kb encoded LSU rRNA. Eucarya are not, in general, sensitive to thiostrepton as they do not contain both of the nucleotides required for interaction. Indeed, base substitution in a synthetic fragment of the mammalian rRNA introducing both sites allows a high affinity binding of thiostrepton (26). In contrast, a universal inhibitor of the peptidyltransferase reaction such as anisomycin interacts with conserved sites in the 3′-half (domain V) of the LSU rRNAs (27). Since the peptidyltransferase site is encoded, as expected, by the nuclear LSU rRNA genes, the 35-kb genome and the linear 6-kb genome (as contiguous fragments 6F and 6G), sensitivity of *P. falciparum* to anisomycin should be predicated by both the organellar- and nuclear-encoded genes (28).

Assay of Inhibition—The effect of thiostrepton on growth and protein synthesis of *P. falciparum* was compared with aniso-

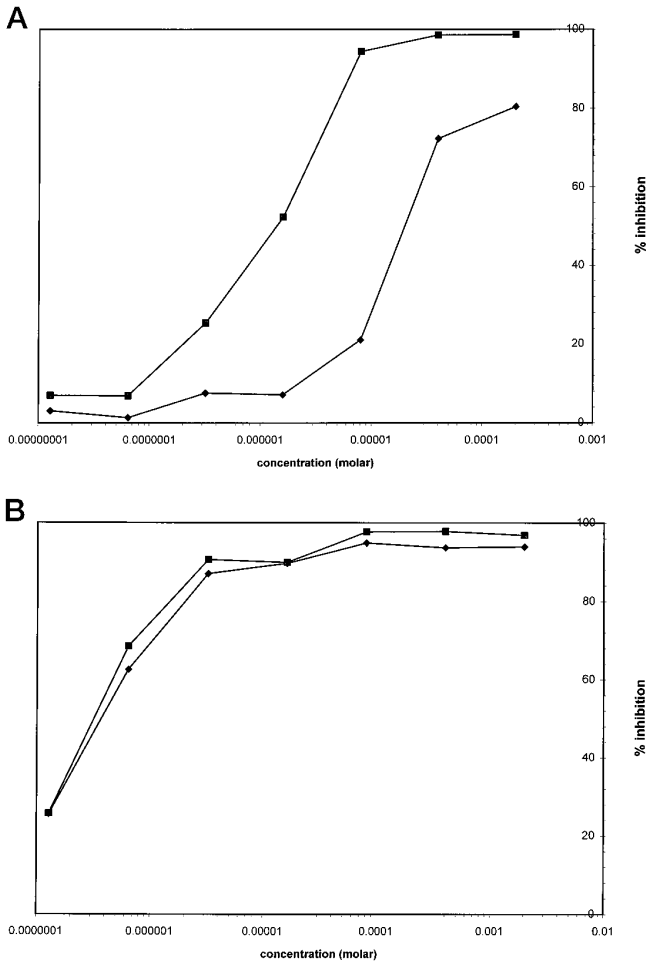

FIG. 2. **Assay of the concentration-response for inhibition of growth (■) and protein synthesis (♦) of *P. falciparum*.** The values obtained, as described under "Experimental Procedures," are expressed as the percent of control cultures. Their difference from 100% is the inhibition, which is plotted *versus* the concentration of drug. Each point is the mean of three determinations. *A*, treatment with thiostrepton; *B*, treatment with anisomycin.

mycin, since the effect of both of these drugs is on protein synthesis. To assay the effect of thiostrepton and anisomycin on the growth of *P. falciparum*, inhibition of the uptake and incorporation of [^{3}H]hypoxanthine was quantitated at serial dilutions of the drugs on an *in vitro* culture of *P. falciparum* (Fig. 2). Both compounds inhibited growth in the micromolar range (IC_{50}, 1.8 μM for thiostrepton and 0.5 μM for anisomycin). These values are comparable with previously published data for anisomycin (28). Inhibition of the incorporation of [^{3}H]leucine was also tested at the same dilutions of the drugs. For anisomycin, total protein synthesis was inhibited 50% at the IC_{50} (0.5 μM) for inhibition of growth, with the concentration-response almost superimposable on that of protein synthesis (Fig. 2*B*). However, for thiostrepton, only a negligible amount of inhibition of total protein synthesis was observed at the IC_{50} for growth. More than 10-fold higher concentrations than the IC_{50} of thiostrepton were required for almost complete inhibition of total protein synthesis. The lack of inhibition of protein synthesis with thiostrepton at the IC_{50} for inhibition of growth suggested that the principal target for the drug is different from cytoplasmic protein synthesis. This would occur if organellar protein synthesis was the target of inhibition (29, 30).

The Target of Inhibition by Thiostrepton—In the absence of a direct measure of plastid-like organellar protein synthesis, assay of mRNA levels by RT-PCR provides a sensitive assay for the selective effect of thiostrepton on the plastid-like organelle. We sought to assay the presence of a protein encoded by the organelle during treatment and would also be targeted by a known inhibitor. One protein that has been identified in the 35-kb genome is a homolog of eubacterial RNA polymerase encoded by the *rpoB* and *rpoC* genes (5). Selective inhibition of the plastid-like RNA polymerase with rifampin provides a comparison with the effect of thiostrepton, since procaryotic RNA polymerases are sensitive to rifampin. The synthesis of the *rpoB/C* mRNA encoded by the 35-kb genome was then compared to a nuclear-encoded mRNA. We took advantage of the fact that the 35-kb encoded *rpoB* and *rpoC* are transcribed as a polycistronic mRNA (3) and assayed nascent transcripts at time points during drug treatment by RT-PCR of the mRNA including the intergenic spacer between *rpoB/C*. Also, the sensitivity of RT-PCR provides a relative estimate of the mRNAs corresponding to those encoded on the 35-kb genome *versus* those of nuclear-encoded mRNAs. Amplification of part of the mRNA corresponding to the merozoite surface antigen (MSA1) gene was chosen as a nuclear-encoded mRNA, as this is abundant in erythrocytic stages of *P. falciparum* (24). As a control, a section of nuclear-encoded small subunit rRNA was also amplified as this is unaffected by antibiotics.

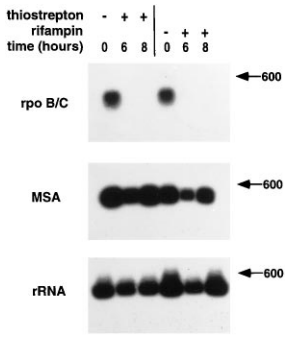

FIG. 3. **Assay of RNA synthesis activity.** Autoradiograph of RT-PCR products from 35-kb- and nuclear-encoded mRNAs from a culture treated with thiostrepton and rifampin, with amplification of total rRNA as a control separated by electrophoresis. The blot was probed corresponding to the RNAs indicated: *rpoB/C*, MSA, and rRNA as discussed. The *left-hand panel* was treated with thiostrepton for the times indicated, and the *right-hand panel* was treated with rifampin. The *arrow* indicates the position of migration of the 600-base pair marker.

The results (Fig. 3) show that thiostrepton and rifampin have similar effects on the decay of mRNA corresponding to *rpoB/C*, occurring within 6 h of drug treatment. A time course with thiostrepton shows a decline of the *rpoB/C* product with time, with a notable effect after only 1 h of treatment (data not shown). Within the duration of the experiment (8 h), the level of *rpoB/C* in untreated controls is consistent (data not shown). Although thiostrepton may be inhibiting a specific subset of nuclear-encoded mRNAs, as the plastid RNA polymerase is composed of subunits of both nuclear and organellar origin, there is no effect on the levels of nuclear-encoded MSA mRNA nor total rRNA. Since completion of the erythrocytic cycle for *P. falciparum* takes about 48 h, it would be expected that the effect on nuclear-encoded mRNAs would be observed only with longer time points and would reflect cell death rather than specific targeting of the plastid-like organelle. These data also support the suggestion that rifampin is a specific inhibitor of the RNA polymerase encoded by the plastid-like organelle (31, 32).

DISCUSSION

We have tested the protein synthesis inhibitors thiostrepton and anisomycin and found that they affect *in vitro* growth and

protein synthesis of *P. falciparum* in different ways. For anisomycin, the inhibition curves for growth and of protein synthesis are almost superimposable over the range of concentrations, showing a direct correlation between inhibiting cytoplasmic protein synthesis and growth of the parasite, as predicted. With thiostrepton, no such correlation exists. At doses of thiostrepton that inhibit growth (IC_{50}, 1.8 μM), there is only a negligible effect on cytoplasmic protein synthesis, and thiostrepton is apparently inhibiting organellar protein synthesis. Both drugs, however, show characteristic dose-response curves for growth inhibition with a specific effect in the micromolar range. Based on considerable evidence from other systems, and the data presented here, the target for thiostrepton is probably the LSU encoded by the 35-kb organelle, while the target for anisomycin is the nuclear-encoded LSU rRNA and perhaps organelle-encoded LSU rRNAs. Although the function of the 35-kb plastid-like organelle is not known (5), inhibition of growth by thiostrepton indicates that protein synthesis from this organelle is essential for growth of the blood stages of the parasite.

More than 10 years ago, an urgent need for drugs against malaria was identified (33). We have investigated the potential of the protein synthesis machinery as a target for drugs. Our approach has been through understanding the primary and secondary structures of the rRNA and testing predictions of defined antibiotics. The antibiotics currently in use, including the tetracyclines and clindamycin, for the treatment and prophylaxis of malaria have little action on pre-erythrocytic stages and slow action on blood stages, but are used for treatment of drug-resistant strains because of their safety rather than their efficacy (34, 35). The target of these drugs has been suggested to be mitochondrial-encoded protein synthesis (28). The IC_{50} of thiostrepton (1.8 μM) and anisomycin (0.5 μM) compare with antimalarial drugs such as pyrimethamine (IC_{50} 8.0–0.08 μM; Ref. 23) and chloroquine (IC_{50} 0.014–0.030 μM; Ref. 24) for sensitive strains (36, 37). However, thiostrepton is sparingly soluble in the *in vitro* culture media for *P. falciparum*, so this may be an overestimate of the IC_{50}. The toxicity of anisomycin precludes the use of this inhibitor *in vivo*, but this study demonstrates the accessibility of the cytoplasmic ribosome and opens the door for searching for more specific analogs. Thiostrepton effectively treats systemic bacterial infections in mice (38) and is also used commercially as a topical treatment of a mixture of antibiotics to treat bacterial infections in animals. Inhibition of *Plasmodium in vivo* by thiostrepton, and other thiazolyl antibiotics, is worthy of investigation in animal model systems. The interaction of thiostrepton with the plastid-like organelle also provides a probe for the function of this organelle, with possible stage-specific effects on erythrocytic stages. Other Apicomplexa may also contain a target for thiostrepton, as the plastid-like organelle from the parasite *T. gondii* encodes rRNAs (39) and the important animal parasite *Babesia bovis* also encodes rRNAs that are probably organellar-encoded (40). Therefore, protein synthesis derived from the plastid-like genome may be a general target for antibiotics in these human and animal parasites, as shown by the selective effect of antibiotics on *T. gondii* (41, 42) and chloroplasts (43).

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