Inhibitors of Nonhousekeeping Functions of the Apicoplast Defy Delayed Death in *Plasmodium falciparum*

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Received 4 July 2006/Returned for modification 6 August 2006/Accepted 25 September 2006

The delayed death phenotype has been rationalized in *Toxoplasma gondii* (2). Treatment of *Toxoplasma* with these drugs does not affect the doubling frequency of these parasites in the first host cell; however, division is slowed upon subsequent invasion of a new host cell. Although this delayed death invoked by the effects of these drugs on apicoplast functions one generation following drug intervention is an interesting and intriguing biological phenomenon by itself, it is a severe limitation where clinical application is concerned. This is especially pertinent in malaria patients, in whom a single cycle of asexual reproduction in *Plasmodium falciparum* takes 48 h to complete and a delay of 48 h or more in treating malaria could have severe consequences for the patient.

The delayed death phenotype has been rationalized in *Toxoplasma* as the consequence of the generation of daughter cells devoid of an apicoplast due to the inability of the apicoplast to segregate following inhibition of an apicoplast function (2, 6). That being so, inhibition of the recently discovered type II fatty acid synthesis pathway in the apicoplast should lead to a similar fate. The antimicrobial biocide triclosan [5-chloro-2-(2,4-dichlorophenoxy) phenol], which targets the enoyl-acyl carrier protein (ACP) reductase of the type II fatty acid biosynthesis pathway, potently incapacitates fatty acid synthesis in this organelle. However, we had observed that triclosan abro-

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gates parasite growth rapidly (29). While this finding indicates a dichotomy in the mechanisms of action of inhibitors of apicoplast functions, it raises several questions as well. In particular, do antibiotics like clindamycin and chloramphenicol, earlier reported to invoke delayed death in Toxoplasma, invoke delayed death in Plasmodium falciparum, too? If they do, does triclosan bring about “rapid death” by virtue of inhibition of fatty acid synthesis per se? If this is true, then does inhibition of the other apicoplast nonhousekeeping metabolic pathways, such as heme biosynthesis, also invoke rapid death? We have endeavored to address these questions here.

**MATERIALS AND METHODS**

**Materials.** The culture medium components RPMI 1640 and HEPES, as well as the reagents, sorbitol, and dimethyl sulfoxide (DMSO), were purchased from Sigma. Triclosan 5000 was obtained from Kumar Organic Products Ltd., Bangalore, India, and its antibacterial activity was tested using Escherichia coli Kan 91 cells prior to its use on Plasmodium culture. Acridine and halosporins were procured from Dr. Ehrenstorfer Chemicals, GmbH. All other inhibitors were obtained from Sigma Chemicals. Inhibitor stocks were made directly either in RPMI 1640 or in DMSO. Stocks were prepared such that the final concentration of DMSO did not exceed 0.05% in the culture medium. Thiolactomycin was a gift from Laurent Kremer, France. R-Lipoic acid and S-lipoic acid were kind gifts of Mulchand Patel (SUNY at Buffalo).

**Intraerythrocytic cultures of Plasmodium falciparum.** For experiments requiring Plasmodium cultures, chloroquine-sensitive, P. falciparum strain FCZ2 (chloroquine sensitive; 50% inhibitory concentration [IC_{50}], 18 nM) was cultivated in type O-positive human erythrocytes in medium supplemented with type O-positive human serum by the candle jar method of Trager and Jensen (31). Cultures were synchronized by 5% sorbitol treatment (13), and parasites were observed for viability and changes in morphology by standard Giemsa staining.

**Determination of death kinetics by microscopy.** To monitor the effects of the various antimalarial compounds on the parasites by microscopy, red blood cells infected with parasites synchronized at the ring stage were cultured in 96-well plates (Nunc, Copenhagen, Denmark) at 10% hematocrit and at an initial parasitemia of ~3%, with a change of medium every 24 h. Inhibitors were added at the required concentrations. The inhibitor concentrations used in the experiments were based on previously published findings as well as by our own observations. All inhibitor additions were done in two sets. In the first set, inhibitor was present in the culture medium throughout the duration of the experiment, i.e., for 96 h. In the second set, inhibitor was present in the culture medium only up to 48 h. Every inhibitor concentration was tested in triplicate. A thin blood smear was prepared every 12 h, and the parasites were observed microscopically in Giemsa-stained smears. The percent parasitemia was calculated from the ratio of the number of infected red blood cells to the total number of red blood cells. Red blood cells were counted in at least 100 fields, each field containing at least 200 cells, using a light microscope (Olympus, Japan). Dead parasites within red blood cells could be differentiated from live cells by the absence of an intact membrane and/or the absence of a stained cytoplasm. Unhealthy parasites, such as those with vacuolation in the cytoplasm, were not counted as dead cells.

**Determination of parasite growth by [3H]hypoxanthine uptake growth inhibition assay (in vitro inhibitor susceptibility assay).** Antimalarial compounds were tested in a cell-based in vitro inhibitor susceptibility assay to determine if they were capable of inhibiting P. falciparum growth. The semiautomated microdilution technique of Desjardins et al., which is based on [3H]hypoxanthine uptake by parasite cultures, was used to assess the sensitivities of the parasites to the selected compounds (1). Briefly, synchronized parasites were cultured in 96-well plates (Nunc) at 2 to 3% hematocrit and at an initial parasitemia of 1 to 2% with various concentrations of inhibitors and with the addition of inhibitor in fresh medium every 24 h. All additions were done in duplicate. For every inhibitor tested, the MIC (MIC_{50}) and IC_{50} were determined in two sets. In the first set, parasites synchronized at the ring stage were cultured in the presence of [3H]hypoxanthine and various concentrations of the particular inhibitor for 48 h. They were then harvested onto glass fiber filters by using a Nunc cell harvester, washed, and subjected to liquid scintillation counting (Hewlett-Packard). In the second set, parasites synchronized at the ring stage were cultured in the presence of various concentrations of inhibitor for the first 48 h. Subsequently, the cultures were incubated with [3H]hypoxanthine for 48 h and harvested. IC_{50} and MIC_{50} were calculated from plots of the relative percent parasitemia versus the log concentration of inhibitor; the concentrations were fitted by nonlinear regression analysis using by Sigma Plot 2000 software. The relative percent parasitemia was calculated as the percent parasitemia of the parasites under treatment and by considering that of untreated parasites to be 100%.

**Determination of change in plastid genome/nuclear genome ratio by quantitative dot blot hybridization.** Parasites were cultured in human red blood cells at 10% hematocrit with or without inhibitor. Parasites were isolated from infected red blood cells by lysis with 0.15% saponin (Sigma) in phosphate-buffered saline (pH 7.4). Total DNA (~2 μg) was extracted from the parasite pellets of control and drug-treated parasite cultures by treatment with proteinase K (Sigma), RNase, and phenol-chloroform extraction and ethanol precipitation. Total DNA was blotted manually, under alkaline transfer conditions, onto a nylon membrane (Hybond N+; Amersham Biosciences). The genes euf (the apicoplast gene that codes for elongation factor Tu) and fabl (the nuclear gene that codes for the enzyme enoyl-ACP reductase) were chosen targets in the apicoplast and nuclear genomes, respectively. Similar-sized probes for these genes were prepared by end labeling the gel-purified PCR products amplified with primers (Microsynth, Switzerland) 5'-CCCCAGATCTATGGAGAAAGAACAAAGATGCATC-3' and 5'-CGTGCTAAGCTTTAATTTTTTATTTCTGTTATAAT-3' for euf with T4 polymerase kinase (GibcoBRL) and a high specific activity [γ-32P]ATP (6,000 Ci/mmol, 5 μCi/ml, NEN). The specific activities of the end-labeled probes measured by liquid scintillation counting (1409 counter; Wallac, San Francisco, CA) were of the order of 10^6 cpm/μg. The blot was hybridized with the probe for euf, washed under stringent conditions, and exposed in a PhosphorImager cassette (FujiFilm Ltd.) by standard procedures. The hybridization blot was then checked for reproducibility. The hybridization probe for euf, and exposed in a PhosphorImager cassette. Probes with similar specific activities were used. The scanned autoradiograms were obtained by using Bio-Rad Quantity One software. The intensity volume (the product of the intensity and the area) of each of the dots was obtained. Apicoplast DNA/nuclear DNA copy number ratios were obtained by calculating the ratios of the intensity volume of the corresponding dots in the autoradiograms hybridized with euf and the dot for fabl. The absolute copy numbers of the apicoplast and nuclear DNA and the plastid genome/nuclear genome ratio were obtained by calculating the ratios of the intensity volume of the corresponding dots in the autoradiograms hybridized with fabl. The absolute copy numbers of the apicoplast genome could not be accurately determined due to differences in the specific activities of the euf and fabl probes. However, relative changes in copy number ratios upon treatment with drugs, which were the parameters of interest in this study, were obtained.

**Competitive PCR to quantitate apicoplast DNA/nuclear DNA ratio in treated Plasmodium falciparum.** Quantitative competitive PCR is based on the competing amplification of a standard template and the gene in the DNA of interest (4). Hence, it is imperative that the standard DNA template have the same primer sites as the gene of interest but that on amplification it produce a PCR product either shorter or longer than the gene in the DNA preparation of interest. For this purpose, the gene of interest is typically cloned in a vector with either an insertion or a deletion within the gene but with the same primers which are to be used for the competitive PCR. The nuclear and plastid probes that we used for determination of the plastid DNA/nuclear DNA ratio were fabl (the nuclear chromosomal gene that codes for the protein enoyl-ACP reductase) and euf (the apicoplast gene that codes for elongation factor Tu). These genes were amplified from total DNA with the primers (Microsynth) 5'-CCCCAGATCTATGGAGAAAGAACAAAGATGCATC-3' and 5'-CGTGCTAAGCTTTAATTTTTTATTTCTGTTATAAT-3'. To perform a quantitation of the plastid DNA copy number ratios upon treatment with drugs, which were the parameters of interest in this study, were obtained.

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both the PCR products were of the same intensity (see Fig. 4). The PCR products were solved by agarose gel electrophoresis and stained with ethidium bromide, and the pET28a/efu/H11001 cation of electrophoresis. There were no significant changes in the efficiencies of amplification of any PCR product from calf thymus DNA, as verified by agarose gel electrophoresis. Competitive PCRs for pET28a/efu/H9004 and pET28a/efu/H9262 were the only detected bands for 5 min and then 25 cycles of 95°C for 1 min, 50°C for 30 s, and 72°C for 45 s, followed by 1 cycle at 72°C for 5 min. The standard DNA templates used for the competitive PCRs for fahb-pGEMT and elfu were various dilutions of fahb-pGEMT and elfu-ET28a+, respectively, in calf thymus DNA. The primers used did not amplify any PCR product from calf thymus DNA, as verified by agarose gel electrophoresis. There were no significant changes in the efficiencies of amplification of fahb-pGEMT and elfu-pGEMT or of elfu-pET28a+ and elfu-pET28a+. The PCR products obtained following competitive PCR were resolved by agarose gel electrophoresis and stained with ethidium bromide, and the amounts of the two PCR products in each case were assessed by densitometry (Bio-Rad Quantity One software). The copy number of the genomic DNA was quantified as the copy number of the standard template in the reaction in which both the PCR products were of the same intensity (see Fig. 4).

Effects of triclosan and clindamycin on incorporation of [35S]methionine into the malaria parasite (Table 1) by morphological examination as well as by [3H]hypoxanthine uptake assay outlined earlier in the presence of 100 μM (the highest noninhibitory concentration of the inhibitors) as determined by [3H]hypoxanthine uptake (data not shown) of R-lipoic acid, S-lipoic acid, the methyl ester of octanoic acid, or the methyl ester of palmitic acid. The IC50 of the inhibitors was calculated in each of these cases.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Enzyme and/or process inhibited</th>
<th>Subcellular location of target</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampin</td>
<td>Prokaryotic RNA polymerase</td>
<td>Apicoplast (predicted)</td>
<td>21</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>Prokaryotic protein synthesis</td>
<td>Apicoplast (predicted)</td>
<td>21</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Prokaryotic protein synthesis</td>
<td>Apicoplast (predicted)</td>
<td>21</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Prokaryotic protein synthesis</td>
<td>Apicoplast, mitochondrion (?)</td>
<td>21</td>
</tr>
<tr>
<td>Halofloxops</td>
<td>Acetyl-CoA carbamoylase, type II fatty acid synthesis</td>
<td>Apicoplast implied by similarity to Toxoplasma</td>
<td>21</td>
</tr>
<tr>
<td>Fluazifloxops</td>
<td>Acetyl-CoA carbamoylase, type II fatty acid synthesis</td>
<td>Apicoplast implied by similarity to Toxoplasma</td>
<td>21</td>
</tr>
<tr>
<td>Quizalofopqs</td>
<td>Acetyl-CoA carbamoylase, type II fatty acid synthesis</td>
<td>Apicoplast implied by similarity to Toxoplasma</td>
<td>21</td>
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<tr>
<td>Cerulenin</td>
<td>Fab B/F, type II fatty acid synthesis</td>
<td>Apicoplast (predicted)</td>
<td>29, 31</td>
</tr>
<tr>
<td>Triclosan</td>
<td>Fab I, type II fatty acid synthesis</td>
<td>Apicoplast (predicted)</td>
<td>29</td>
</tr>
<tr>
<td>Succinyl acetone</td>
<td>Δ-Aminolevulinic acid dehydratase, heme synthesis</td>
<td>Apicoplast (predicted)</td>
<td>28</td>
</tr>
<tr>
<td>Acifluorfen</td>
<td>Protoporphyrinogen oxidase, heme synthesis</td>
<td>Mitochondrion implied by similarity to Toxoplasma</td>
<td>16, 21</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>Eukaryotic protein synthesis</td>
<td>Cytosol (predicted)</td>
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</tr>
<tr>
<td>Chloroquine</td>
<td>Heme polymerization</td>
<td>Food vacuole</td>
<td>24</td>
</tr>
<tr>
<td>Radicicol</td>
<td>Hsp90, ATP citrate lyase implied</td>
<td>Cytoysl</td>
<td>11, 26</td>
</tr>
</tbody>
</table>

* Acetyl-CoA, acetyl coenzyme A.

We first examined the kinetics of death invoked by inhibitors of various processes occurring in different subcellular organelles of the malaria parasite (Table 1) by morphological examination as well as by [3H]hypoxanthine uptake. Treatment with 5 μM clindamycin or 50 μM chloramphenicol did not produce a significant decrease in parasitemia during the first cycle of asexual reproduction of the culture. However, parasitemia decreased sharply at about 48 h (Fig. 1A), which coincides with the late trophozoite stage of the second cycle of asexual reproduction (Fig. 1A). Importantly, the trend remained the same whether the cultures were incubated with the antibiotics for this experiment were chosen to be manyfold higher than those required for inhibition of parasite growth, yet no effect was seen at 48 h. Lower concentrations of these antibiotics (Fig. 1A) also brought about a similar effect (data not shown). This confirmed the delayed actions of these antibiotics on parasite growth. Treatment with 50 μM tetracycline and 5 μM rifampin also resulted in a sharp decrease in parasitemia only at about 84 h, although there was a modest decrease in the parasitemia during the first cycle, also. This effect in the first cycle is probably due to inhibition of prokaryotic protein synthesis in the mitochondrion, in addition to that in the apicoplast. Lower concentrations of tetracycline and rifampin also inhibited parasite growth only in the second cycle, without affecting growth at 48 h (data not shown). That tetracycline and rifampin did bring about delayed death was also clear from the growth inhibition curves, which indicated that the concentrations of these compounds required to inhibit parasite growth at 48 h and 96 h were significantly lower.
different (Fig. 1B; Table 2). Therefore, the primary target of these compounds seemed to be the apicoplast. Figure 1C depicts the differences in the growth of parasites treated for 48 h and for 96 h with clindamycin, chloramphenicol, rifampin, and tetracycline at their MIC90s, as determined by [3H]hypoxanthine uptake. While these inhibitors reduced the parasitemia level to 10% after 96 h, they did not have much of an effect on the parasites at 48 h, thus reiterating delayed death as the mode of death induced by these antibiotics. Thus, inhibition of an apicoplast process such as protein synthesis does bring about delayed death in *Plasmodium falciparum*, too, as in *Toxoplasma gondii*.

On the contrary, agents with nonapicoplast targets, such as chloroquine, amodiaquine, clotrimazole, radicicol, cycloheximide, glyphosate, DPI, and acifluorfen, as well as agents like triclosan, thiolactomycin, cerulenin, NAS-91, and the fops series of inhibitors, which target fatty acid synthesis in the apicoplast, and succinyl acetone, which inhibits heme biosynthesis in this organelle, invoked death itself at about 36 h (Fig. 1A), which corresponds to the trophozoite stage of the first cycle of asexual reproduction. Parasites incubated with these inhibitors were killed before the culmination of the first cycle of asexual reproduction, and hence, there was no reinvasion of new host cells. The IC50s determined for cultures incubated with these agents for 48 and 96 h showed no statistically significant difference (Student’s *t* test, *P* < 0.01), indicating the absence of delayed death in treatments with these agents (Fig. 1B; Table 2). The percent parasitemias of cultures treated with these inhibitors at their MIC90s for 48 h and 96 h were the same (~10%), thus confirming rapid death (Fig. 1C).

Inhibitors of fatty acid synthesis and heme synthesis therefore seem to elicit rapid death. However, the ultimate proof of rapid death rests with the demonstration of an unaltered apicoplast genome copy number relative to the nuclear genome copy number, i.e., no loss of the apicoplast. We used quantitative hybridization and competitive PCR to determine the apicoplast genome copy number (and, hence, to determine the presence of the apicoplast) in control and treated cultures.

We used quantitative hybridization analysis to examine the reduction, if any, in the copy number of the apicoplast genome upon treatment with clindamycin and chloramphenicol and also to confirm the absence of the delayed death phenotype with triclosan treatment. As shown in Fig. 2, the apicoplast genome is present at ~0.91 copies per haploid nuclear genome in the untreated parasites. On treatment with clindamycin, the plastid genome copy number was specifically reduced to ~0.65 copies after 48 h and was further reduced to ~0.42 copies after 96 h, indicating that the delayed death invoked by clindamycin treatment occurs subsequent to apicoplast loss. The partial apicoplast effect after 48 h of treatment with clindamycin which we observed is similar to that reported earlier for *Toxoplasma gondii* (2). It is expected that with continuous treatment with clindamycin, parasites that did not lose the apicoplast in the first 48 h (probably either due to the inherent efficacy of clindamycin or due to the absence of absolute synchrony during clindamycin treatment) would do so by 96 h, and hence, the effect at 96 h would be greater than that at 48 h.

Treatment with triclosan, however, showed a decline in both nuclear and plastid genome copy numbers in parallel, indicating that the parasites per se were ablated within the first cycle
of growth itself. These data are suggestive of a fundamental difference in the mechanisms of action of triclosan and clindamycin and emphasize our observation that triclosan invokes rapid death and not delayed death in the malaria parasite, even though each protein targets an enzyme of the apicoplast.

While the data obtained upon quantitative hybridization analysis did provide biochemical proof for the loss of the apicoplast in clindamycin-treated parasites, it typically suffers from the disadvantages of the unequal specific activities of the probes and errors that arise from the unequal transfer and incomplete stripping of the probe. We therefore decided to accurately determine the absolute apicoplast copy numbers using quantitative competitive PCR. DNA from parasites treated for 72 h with triclosan, clindamycin, chloroquine, haloxyfops, succinyl acetone, NAS-91, cerulenin, DPI, cycloheximide, and tetracycline were subjected to quantitative competitive PCR. As shown in Fig. 3, while treatment with clindamycin and tetracycline attenuated the apicoplast copy number significantly from 1.0 to 0.4 and 0.1, respectively, treatment with triclosan, chloroquine, haloxyfops, succinyl acetone, NAS-91, cerulenin, DPI, and cycloheximide did not alter the ratio of the apicoplast genome copy number to the nuclear genome copy number. The significant difference between the apicoplast effect obtained following treatment with clindamycin and tetracycline could be due to the differences in the levels of the drugs with respect to their IC50s.

We monitored the incorporation of [1,2-14C]acetate into fatty acids in cultures treated with clindamycin to check whether inhibition of the housekeeping functions of the apicoplast leads to inhibition of fatty acid synthesis in the first cycle of asexual reproduction and to confirm apicoplast loss and the consequent absence of the fatty acid synthesis pathway in the second cycle of asexual reproduction. While the inhibitor of FabI, triclosan, inhibited fatty acid biosynthesis, as determined by the decreased level of incorporation of [1,2-14C]acetate into fatty acids in cultures treated with clindamycin (Fig. 4). In the second cycle of asexual reproduction, however, very little incorporation of [1,2-14C]acetate into fatty acids was seen in clindamy-

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC50 (µM) (error) at:</th>
<th>MIC90 (µM) (error) at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48 h</td>
<td>96 h</td>
</tr>
<tr>
<td>Cerulenin</td>
<td>15.1 (1.1)</td>
<td>16.9 (1.1)</td>
</tr>
<tr>
<td>Fluazifops</td>
<td>1,548.8 (110)</td>
<td>1,479.1 (1150)</td>
</tr>
<tr>
<td>Haloxyfops</td>
<td>107.1 (10.9)</td>
<td>97.7 (10.3)</td>
</tr>
<tr>
<td>Quizalofops</td>
<td>575.4 (51.8)</td>
<td>676.1 (40.2)</td>
</tr>
<tr>
<td>Succinyl acetone</td>
<td>17,782.8 (1,102.3)</td>
<td>17,378.0 (1,092.4)</td>
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<td>Triclosan</td>
<td>1.1 (0.01)</td>
<td>1.2 (0.1)</td>
</tr>
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<td>Chloramphenicol</td>
<td>416.8 (10.3)</td>
<td>11.2 (1.2)</td>
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<tr>
<td>Clindamycin</td>
<td>288.4 (21.8)</td>
<td>0.0013 (0.0005)</td>
</tr>
<tr>
<td>Rifampin</td>
<td>4.2 (0.12)</td>
<td>0.19 (0.011)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>134.9 (10.5)</td>
<td>2.19 (0.1)</td>
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<tr>
<td>Acifluorfen</td>
<td>524.8 (31.2)</td>
<td>457.1 (40.3)</td>
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<td>Chloroquine</td>
<td>0.0048 (0.0015)</td>
<td>0.0042 (0.0011)</td>
</tr>
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<td>Cycloheximide</td>
<td>0.15 (0.01)</td>
<td>0.10 (0.03)</td>
</tr>
<tr>
<td>Radicicol</td>
<td>7.2 (1.2)</td>
<td>5.6 (0.7)</td>
</tr>
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</table>
cin-treated parasites, indicating apicoplast loss and a consequent absence of fatty acid synthesis (Fig. 4).

We also monitored the effect of lipoic acid on growth inhibition of the parasites by triclosan and chloramphenicol. Lipoic acid neither delayed the rapid death invoked by triclosan nor reversed the inhibition by chloramphenicol (Fig. 5).

**DISCUSSION**

Within a couple of years of the discovery of the apicoplast, Fichera and Roos demonstrated that the apicoplast is indispensable to the apicomplexan parasite *Toxoplasma* (2). Antibiotics that incapacitate the housekeeping functions of DNA replication, transcription, and protein synthesis of this organelle interfere with the replication of the organelle and, consequently, with its segregation during schizogony. Transient mutants that are incapable of replicating the apicoplast also share the same fate (6). Although *Toxoplasma* and *Plasmodium* have similar life cycles, while each infectious 48-h erythrocytic asexual cycle of *Plasmodium* involves only one schizogony cycle, followed by rupture of the host red blood cell, and while the merozoites generated during every schizogony cycle must invade a new host cell to continue the asexual stage of the life cycle, in *Toxoplasma* the 48-h asexual cycle comprises several 7-h doublings of a tachyzoite within the same host cell, followed by host cell lysis. Daughter cells of *Toxoplasma* with compromised apicoplasts are able to survive while they remain in the same host cell. However, even though they appear to be healthy and grow at a normal rate, they are unable to successfully establish a new infection and, hence, die a delayed death. Exactly what causes the delayed death phenomenon following the loss of apicoplast function still remains a mystery. It has been postulated that the apicoplast is required for replenishing the reserves of a resource presumably involved in the generation of the parasitophorous vacuole that surrounds the parasite in the host cell and is crucial to a successful host cell invasion (2, 22). In the event of such a scenario, identification of the key molecules that are synthesized by the apicoplast and that obviate a rapid death would provide insight into understanding the phenomenon of delayed death. Most of the predicted apicoplast-targeted proteins with anabolic func-
progress to the schizont stage. This discrepancy in the actions of these antibiotics on *Toxoplasma* and *Plasmodium* probably stems from the occurrence of the cytosolic type I fatty acid biosynthesis pathway in *Toxoplasma* (22). It is possible that in the presence of a functional cytosolic fatty acid synthesis pathway, fatty acids synthesized by the type II fatty acid synthesis pathway resident in the plastid in *Toxoplasma* are specifically required during the process of host cell invasion. In contrast, *Plasmodium* harbors only a type II fatty acid synthesis pathway in the apicoplast, which therefore must minister to the lipid requirement of not just host cell invasion but other processes as well. Subsequently, apicoplast loss following treatment of *Plasmodium* with delayed death-invoking agents kills the parasites in the trophozoite stage of the second asexual cycle, as observed experimentally. Importantly, treatment of *Plasmodium* with inhibitors of fatty acid synthesis or heme synthesis leads to rapid death and not delayed death.

Our findings pose a question, however. If the fatty acid and heme biosynthesis pathways are so crucial to the survival of *Plasmodium* that parasites treated with inhibitors of the fatty acid and heme biosynthesis pathways do not survive to invade the next host cell, how do parasites treated with delayed death-invoking agents survive almost an entire cycle without a functional apicoplast (and, hence, a functional fatty acid synthesis or heme synthesis pathway)? This apparent paradox is resolved when it is recalled that fatty acid synthesis occurs maximally in the metabolically active trophozoite stage (29). The fatty acids synthesized in the trophozoite stage are crucially required during the trophozoite stage and are probably sufficient to last the parasite until the following invasion by the merozoite. Inhibition of fatty acid synthesis in the trophozoite stage by an inhibitor of fatty acid synthesis generates a paucity of fatty acids. The trophozoite stage fails to progress to the schizont stage, and the parasite dies a rapid death. However, all proteins involved in the functioning of the fatty acid, heme, and isoprenoid biosynthesis pathways are nucleus encoded and apicoplast targeted, and inhibition of apicoplast DNA, RNA, or protein synthesis (by clindamycin) does not interfere with fatty acid biosynthesis in the apicoplast in the first cycle of asexual reproduction (Fig. 4). Consequently, during the first 48 h of treatment with a delayed death-invoking agent, fatty acids are still synthesized, despite the inhibition of apicoplast DNA replication, transcription, or protein synthesis. This allows the survival and invasion of the next host cell. In the second cycle, in the absence of an apicoplast, fatty acids are not synthesized during the trophozoite stage of the malaria parasite, and hence, they are unable to survive and progress to the schizont stage.

In lieu of our observation that the apicoplast ministers to the parasite’s requirement of lipids essential not just for host cell invasion but also during the metabolically active trophozoite stage itself, we wondered if lipoic acid, a cofactor of mitochondrial α-keto acid dehydrogenase, which is synthesized in the apicoplast, is the “key” molecule synthesized by the apicoplast. Lipoic acid is an efficient free radical scavenger and plays a pivotal role in proffering protection against oxidative insults (19, 36). In fact, Toler suggests that the apicoplast in apicomplexans, by virtue of its ability to synthesize lipoic acid, was retained as an obligate endosymbiont under evolutionary selection pressure to combat the oxidative injury generated by...
mitochondrial reactive oxygen species during pyrimidine biosynthesis in the schizont stage (30). Lipoic acid is produced from octanoyl-ACP and cysteine by the apicoplast resident lipoic acid synthase (LipA). Furthermore, synthesis of the enzyme LipA, which utilizes an Fe-S cluster, is dependent on parasite heme biosynthesis. Consequently, inhibition of fatty acid synthesis or heme synthesis should deprive the parasite of lipoic acid. If parasite survival hinged on lipoic acid reserves, then the exogenous addition of lipoic acid would enable the parasite to overcome fatty acid or heme synthesis inhibition. However, the results of our experiments demonstrate that the externally administered lipoic acid is not capable of rescuing triozolan-treated parasites from rapid death (Fig. 5). Lipoic acid also could not rescue chloramphenicol-treated parasites from delayed death (Fig. 5). Our findings suggest that Toler’s hypothesis (30) is an oversimplified one. While lipoic acid could be one of the essential molecules synthesized by the apicoplast, the apicoplast, in all probability, does not exist for the synthesis of one key molecule but exists for the synthesis of various molecules, many of which are essential for parasite survival.

Our study unequivocally demonstrates that although drugs which interfere with the processes of apicoplast replication, transcription, and translation lead to apicoplast loss, they do not kill the malaria parasite rapidly, as they permit other apicoplast biochemical processes essential to the survival of the parasite to proceed, thus enabling it to survive a cycle of growth. However, inhibitors of fatty acid biosynthesis and heme biosynthesis, which are essential functions of the apicoplast, like the processes mentioned above, appear to impinge more critically on the very survival of the parasite. These inhibitors kill the parasite as rapidly as inhibitors or drugs that work on targets outside the apicoplast. Thus, the machinery within the apicoplast appears to serve two distinct but related functions, i.e., “self-sustenance” and the “sustenance of the organism” as a whole. The observation of this dichotomy provides hope for the further development of drugs which inhibit processes other than those intimately linked with the self-sustenance of the apicoplast per se. Inhibitors of fatty acid synthesis, heme biosynthesis, and probably, isoprenoid biosynthesis, too, could very well be our best bets as therapeutic agents that can be used to combat malaria rapidly and effectively.

ACKNOWLEDGMENTS

This work was supported by a grant from the Department of Biotechnology (DBT), Government of India, to N.S. and A.S. A.S. is also generously supported by a grant from the Centre of Excellence, DBT. T.N.C.R. acknowledges the Council of Scientific Industrial Research, Government of India, for a senior research fellowship. S.M. acknowledges DBT for a postdoctoral fellowship. We thank Mulchand Patel for the kind gifts of R-lipoic acid and γ-lipoic acid.

We do not have any competing financial interests with regard to this work.

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