Azithromycin (AZ), a broad-spectrum antibacterial macrolide that inhibits protein synthesis, also manifests reasonable efficacy as an antimalarial. Its mode of action against malarial parasites, however, has remained undefined. Our in vitro investigations with the human malarial parasite *Plasmodium falciparum* document a remarkable increase in AZ potency when exposure is prolonged from one to two generations of intraerythrocytic growth, with AZ producing 50% inhibition of parasite growth at concentrations in the mid to low nanomolar range. In our culture-adapted lines, AZ displayed no synergy with chloroquine (CQ), amodiaquine, or artesunate. AZ activity was also unaffected by mutations in the *pfCRT* (*P. falciparum* chloroquine resistance transporter) or *pfdnrt* (*P. falciparum* multidrug resistance-1) drug resistance loci, as determined using transgenic lines. We have selected mutant, AZ-resistant 7G8 and Dd2 parasite lines. In the AZ-resistant 7G8 line, the bacterial-like apicoplast large subunit ribosomal RNA harbored a U438C mutation in domain I. Both AZ-resistant lines revealed a G76V mutation in a conserved region of the apicoplast-encoded *P. falciparum* ribosomal protein L4 (PfRpl4). This protein is predicted to associate with the nuclear genome-encoded *P. falciparum* ribosomal protein L22 (PfRpl22) and the large subunit rRNA to form the 50 S ribosome polyepptide exit tunnel that can be occupied by AZ. The PfRpl22 sequence remained unchanged. Molecular modeling of mutant PfRpl4 with AZ suggests an altered orientation of the L75 side chain that could preclude AZ binding. These data imply that AZ acts on the apicoplast bacterial-like translation machinery and identify *Pfrpl4* as a potential marker of resistance.

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Another antibiotic that appears particularly promising for antimalarial combination therapy is azithromycin (AZ), a 15-membered azalide that has been broadly used for the treatment of streptococcal, staphylococcal, chlamydial, and gonorrheal infections. Notably, AZ displays a good safety profile for young children and pregnant women (6). This semisynthetic second-generation antibiotic was derived from its parent compound erythromycin by introducing a methyl-substituted nitrogen group at position C-9 of the lactone ring, resulting in several improved features. These include a broader spectrum of activity, more favorable pharmacodynamics, and a longer elimination half-life. This extended half-life has been attributed to AZ concentrating inside lysosomes in phagocytic cells, as a result of protonation of its two basic amine groups (7), followed by AZ leaching out into the bloodstream over a period of several days to a week. This provides effective therapeutic coverage with AZ for several days post-completion of treatment.

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**In Vitro Efficacy, Resistance Selection, and Structural Modeling Studies Implicate the Malarial Parasite Apicoplast as the Target of Azithromycin**

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proteins L4 and L22 that line the protein exit tunnel (8–10). AZ binding interferes with transpeptidation/translocation of nascent polypeptides exiting the polypeptide tunnel, leading to premature detachment of incomplete peptide chains and subsequent cell death (11–13).

AZ activity against malarial parasites was initially discovered by \textit{in vitro} testing of chloroquine (CQ)-sensitive and CQ-resistant \textit{P. falciparum} lines (14, 15). Further studies revealed a slow onset of parasite killing, similar to tetracycline, doxycycline, and clindamycin (16). \textit{In vitro} screening for potential drug combinations was suggestive of additive to synergistic interactions with AZ-CQ as well as AZ-quinine combinations in some CQ-resistant \textit{P. falciparum} strains (17). Against CQ-sensitive strains, the effect was additive. \textit{In vivo} studies, initially conducted in mice infected with blood stage \textit{Plasmodium berghei} parasites, also showed good treatment efficacy with AZ monotherapy. This efficacy was enhanced by combining AZ with the fast-acting blood schizonticidal agents CQ, quinine, artemisinin, or halofantrine (15, 18). AZ curative blood schizonticidal efficacy was also confirmed in \textit{Aotus} monkeys infected with CQ-resistant \textit{P. falciparum} (18). Evidence that AZ was clinically effective in treating \textit{P. falciparum} malaria in humans came from pilot studies showing that AZ monotherapy was an effective prophylactic agent (19, 20). A more recent treatment trial in India reported close to 100% cure rates in patients treated with AZ plus CQ, with this combination proving to be far more effective than monotherapy with the individual agents (21). High levels of treatment efficacy were also observed in a recent trial from Thailand that studied regimes of combining AZ with quinine or artesunate (22). A monotherapy trial in India also found AZ to be well tolerated and highly effective in treating \textit{Plasmodium vivax} malaria (23).

The proven antimalarial efficacy observed with AZ has made it important to define its mode of action against malarial parasites. One pressing question is whether AZ, a weak base, acts inside the parasite digestive vacuole (DV), a lysosomal-like acidic compartment (pH 5.0–5.4) where CQ and other weak base 4-aminoquinolines concentrate and act by interfering with heme detoxification (24–26). Such an activity might account for earlier reports of AZ-CQ \textit{in vitro} synergy (17). Another possibility is that AZ acts by inhibiting the bacterial-like protein synthesis machinery present in the \textit{Plasmodium} apicoplast, an organelle of cyanobacterial origin that has been shown in the related Apicomplexan parasite \textit{Toxoplasma gondii} to be indispensable for parasite growth (27–29). To address these questions, we have investigated the efficacy of AZ alone or in combination with fast-acting antimalarials, the effect on AZ susceptibility of point mutations in DV transmembrane transporters, the selection of AZ-resistant parasite lines, and the molecular mechanisms that can cause AZ resistance. Our results directly implicate the apicoplast 50S ribosomal subunit as the target of AZ in \textit{P. falciparum}.

**EXPERIMENTAL PROCEDURES**

**Parasite Culture and \textit{in Vitro} Antimalarial Drug Assays—** \textit{P. falciparum} parasites were cultured \textit{in vitro} in human red blood cells as described (30). Antimalarial susceptibilities were measured in 96-well plates over 48 or 96 h of exposure with an initial parasitemia of 0.5 or 0.2% synchronized ring stage parasites, respectively. Drug IC$_{50}$ values (producing 50% inhibition of [$^{3}$H]hypoxanthine uptake) were derived by linear regression from the dose-response curves (30, 31). In the 48-h drug assays, parasites were exposed to drug for 30 h, and 0.5 $\mu$Ci of radiolabeled [$^{3}$H]hypoxanthine was added to each well for the last 18 h. In the 96-h drug assays, media was replaced after 48 and 72 h, and [$^{3}$H]hypoxanthine was added at the 72-h time point (0.5 $\mu$Ci/well). Media replacements contained appropriate amounts of drug to ensure that drug concentrations remained constant in each well throughout the duration of the assay. To test the interaction of AZ with other drugs, each drug was tested alone and in combination at fixed ratios of its IC$_{50}$ value, as calculated separately for each assay (AZ, test drug ratios of 0:1, 1:3, 1:5, 1:1, 3:1, 5:1 and 1:0). Fractional IC$_{50}$ (FIC$_{50}$) values from 96-h assays were calculated as previously described (32) and used to plot isobolograms. Two-tailed unpaired Student’s $t$-tests were performed for statistical comparisons. AZ dihydrate (CP-62,993-03) was obtained from Pfizer. Thiostrrepton, tetracycline, doxycycline, and erythromycin were purchased from Calbiochem, whereas triclosan and CQ were purchased from Sigma.

**Selection of AZ-resistant \textit{P. falciparum} Lines—** \textit{P. falciparum} lines were selected for resistance to AZ using the following regime. First, we exposed Dd2 and 7G8 parasites ($5 \times 10^{10}$ each) to 38 and 115 nm AZ, respectively (i.e. 30 and 90 ng/ml of AZ dihydrate), for 3 days with twice daily feeding. We then increased the drug concentration to 380 nm for both lines and maintained this level for 4 days. For the following 15 days Dd2 and 7G8 parasite lines were again maintained at 38 and 115 nm AZ, respectively. Giemsa-stained thin blood films were examined every 2 to 3 days to check for viable asexual-stage parasites. Once a week, 30–40% of the culture was replaced with freshly drawn red blood cells. For both lines, AZ-resistant ring stage parasites were observed by day 21. Once the parasitemias reached 2–3%, parasites were phenotypically characterized for their drug response profiles using [$^{3}$H]hypoxanthine incorporation assays. Dd2 and 7G8 AZ-resistant parasite lines (termed AZ-R$^{Dd2}$ and AZ-R$^{7G8}$, respectively) were subsequently cloned by limiting dilution (33) and cultured in the presence of 640 and 1280 nm AZ, respectively.

**DNA Constructs and Sequencing—** A 1.0-kb DNA fragment encompassing the open reading frame of the \textit{P. falciparum} apicoplast-encoded ortholog of the \textit{rpl}$^{4}$ ribosomal protein gene (denoted \textit{Pfrp}$^{4}$; GenBank\textsuperscript{TM} accession number X95276) was PCR amplified from the wild-type and AZ-resistant parasite lines using primers p1083 (5’-AATGGTAACATATCTATTT-TGGGAAATAG) and p1084 (5’-AATTTCCACCTTCTT-TATTTAATAGTA). This fragment was used in nested PCR with internal primers p1112 (5’-TTTCTGAATATTAT-TATTTAAAATTAATACCA; Clal site in lowercase) and p1111 (5’-AcgtagaATTTAAAAATTATTTAACATATT-ATATCGACGTTTC) (BsiWI site in lowercase) to amplify a full-length 0.6-kb coding sequence fragment lacking only the ATG start and TAA stop codons. Amplified products were cloned into pCR2.1-TOPO vector (Invitrogen) and sequenced using T7 and M13-reverse primers (Invitrogen).
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For the transfection studies, sequence-verified PfPrl4 inserts were subcloned into the ClaI and BsiWI restriction sites of the expression plasmid pLN-ACP-V5. This plasmid, based on pLN (34), contains a 180-bp sequence encoding the N-terminal bipartite leader sequence of the fatty acid synthase acyl-carrier protein (ACP), used to target proteins to the apicoplast (35), as well as a 3’ sequence encoding a C-terminal V5 epitope tag (GKPIPNPLLGLDST). The final construct, termed pLN-ACP-Pfrpl4-V5 (supplemental Fig. 1), contained mutant or wild-type PfPrl4 fused at its 5’ end to the ACP leader sequence and at its 3’ end to the V5 tag. This fusion gene is regulated by a 1.0-kb calmodulin 5’-untranslated region and a 0.8-kb hsp86 3’-untranslated region. Related constructs were made that replaced the 3’ V5 tag with a GFP-Mut2 (green fluorescent protein) sequence, creating the pLN-ACP-Pfrpl4-GFP plasmid. From these plasmids, we derived pLN-SP-Pfrpl4-V5 and pLN-SP-Pfrpl4-GFP by replacing the full-length ACP apicoplast targeting sequence with a 57-bp signal peptide (SP) sequence from the ACP gene. All of these constructs were also made with a 0.9-kb hsp86 5’-untranslated region fragment in place of the calmodulin 5’-untranslated region. This resulted in 16 constructs, which were all transfected into Dd2 parasites. The calmodulin promoter series of constructs were also transfected into 3D7 and GC03 parasites. Transfections were carried out as previously described (36) and recombinant lines were selected using 2.5 μg/ml blasticidin hydrochloride.

The 0.9-kb full-length P. falciparum nuclear genome-encoded ortholog of the rpl22 ribosomal protein gene (PfPrl22; PlasmoDB ID number PF14_0642) was PCR amplified using p1429 (5’-TAATGATAATTGCGGAATGTGTTTGT and p1432 (5’-GTACATAAATAATATACACATATTATTTAAAAACAGGA). The apicoplast large subunit (LSU) rRNA gene (GenBank accession number X95275) was PCR amplified as a 2.9-kb fragment with primers p986 (5’-GCGAAATAGAGATAAAGGGAAATTCG) and p800 (5’-AGCTAATGGTTGAGATTGAGT). PCR fragments were gel purified and fully sequenced on both strands using internal primers.

RESULTS
AZ Displays Extraordinary Increases in Potency against P. falciparum Strains Exposed for a Second Generation of Growth as Compared with Thioridazone, Triclosan, and CQ—A previous study with two P. falciparum lines observed an 80–220-fold increased potency when the duration of AZ exposure was prolonged from 48 to 96 h (16). We extended this observation to other multidrug-resistant and drug-sensitive lines and compared the effect to that of other antimalarial agents that share similar modes of action or are in clinical use. The lines chosen were Dd2 (Indochina; resistant to CQ, quinine, pyrimethamine, and sulfadoxine), 7G8 (Brazil; resistant to CQ and pyrimethamine), and GC03 (a CQ-sensitive progeny of the HB3 × Dd2 genetic cross (40)). Comparative antimalarial agents included thioridazone (which targets the GTPase site of the apicoplast LSU rRNA (41, 42)); triclosan (a topical antimicrobial that inhibits enoyl-ACP reductase, a component of the fatty acid type II biosynthesis pathway in the Plasmodium apicoplast (43, 44)), and CQ (which binds to heme moieties in the parasite DV and prevents their detoxification (45)). A comparative profile of responses to each compound for these parasite lines is presented as IC50 values in Fig. 1. After 48 h, Dd2, 7G8, and GC03 displayed AZ IC50 values of 3.5, 15.7, and 18.1 nM, respectively, whereas after 96 h these values decreased to 103, 190, and 77 nM, respectively (supplementary Table 1). This translated into a 4.5-, 1.3-, and 2.5-fold increases in susceptibility. In 96-h assays with these same lines, the triclosan IC50 values were 4186, 424,
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**FIGURE 2. Isobologram plots of AZ plus CQ tested against P. falciparum culture-adapted lines.** FIC$_{50}$ values were determined for AZ and CQ tested at different concentration ratios (0:1, 1:3, 1:5, 1:1, 3:1, 5:1, and 1:0). Values for the Dd2, 7G8, and 3BA6 lines were calculated from 96-h assays, whereas for the TM90C6A and TM90C6B lines these were calculated from 72-h assays. For each line, isobolograms were performed at least 5–7 times for each drug combination.

and 1526 nM, and CQ IC$_{50}$ values were 134, 113, and 24 nM, respectively. This corresponded to 1.0–2.0-fold increases in tri-closan or CQ susceptibility. These results indicate that AZ becomes a highly active antimalarial that manifests its potency after a second generation of parasite intraerythrocytic growth. This increase in potency far exceeds other agents that also presumably target apicoplast pathways, including in the case of thiostrepton presumably the same ribosomal subunit involved in protein synthesis.

**AZ Is Not Synergistic with CQ, Amodiaquine, or Artesunate—** To investigate the antimalarial activity of AZ in combination with CQ, 96-h drug assays were performed using the CQ-resistant *P. falciparum* lines Dd2, 7G8, and 3BA6 (the latter is a CQ-resistant progeny of the HB3 × Dd2 cross). From these, FIC$_{50}$ values were determined and isobolograms were derived and plotted. As illustrated in Fig. 2, this combination appeared essentially additive for Dd2 (mean FIC$_{50}$ value: 1.39, 95% confidence interval (CI), 1.10–1.69) and 3BA6 (mean FIC$_{50}$ value: 1.38, 95% CI, 1.22–1.55) and was also additive for 7G8 (mean FIC$_{50}$ value: 1.05, 95% CI, 1.02–1.08).

We also assayed the TM90C6A and TM90C6B Thai isolates that have been more recently adapted to *in vitro* culture (46). AZ-CQ combination assays performed with these lines also found no evidence for synergy (TM90C6A mean FIC$_{50}$ value, 1.35, 95% CI, 1.25–1.45; TM90C6B mean FIC$_{50}$ value, 1.15, 95% CI, 1.04–1.25; Fig. 2).

In view of the decline of CQ as a first-line antimalarial and the increasing interest in the use of amodiaquine or artemunate as alternative antimalarials (notably for combination therapy), we also assayed AZ-amodiaquine and AZ-artesunate combinations with the drug-resistant Dd2 and 7G8 lines. In 96-h combination assays, mean FIC$_{50}$ values for AZ-amodiaquine were 1.28 (95% CI, 1.01–1.56) and 1.23 (95% CI, 0.87–1.58), whereas mean FIC$_{50}$ values for AZ-artesunate were 1.62 (95% CI, 1.02–2.22) and 1.34 (95% CI, 1.14–1.54) against Dd2 and 7G8 parasite lines, respectively (data not shown). Thus, these assays found no evidence of *in vitro* synergy between AZ and either of these drugs.

**Parasite Susceptibility to AZ Is Not Influenced by Mutations in the DV Transmembrane Proteins PfCRT and PfMDR1—** We assessed whether *P. falciparum* susceptibility to AZ, *in vitro* could be influenced by point mutations in the DV transmembrane proteins PfCRT or PfMDR1 (the latter is also known as Pgh1). This was for several reasons. First, genetic studies have demonstrated that mutant forms of these proteins can affect parasite *in vitro* susceptibility to a wide range of antimalarials, including CQ, quinine, mefloquine, halofantrine, and the anti-influenzal agent amantadine (47–53). Second, there is a published report of AZ-CQ synergism that raised the possibility of their sharing a related mode of action (17). Third, AZ, like CQ, is a weak base that could be predicted to accumulate in the acidic *P. falciparum* DV (where CQ also accumulates and exerts its antimalarial property (54)). We thus took advantage of existing recombinant lines that had been genetically modified to express mutant forms of either protein (50, 52, 55) and tested these in 96-h assays with AZ. Results showed AZ IC$_{50}$ values in the range of 48–194 nM for *pfCRT* or *pfMDR1* genetically modified lines, with no significant changes observed between these lines and their parental or recombinant control lines (Fig. 3; haplotypes and IC$_{50}$ values are reported in supplemental Table 2). These results demonstrate that mutations in these DV transporter proteins do not noticeably modulate parasite susceptibility to AZ.

**AZ-resistant P. falciparum Lines Can Be Selected In Vitro—** To assess whether AZ resistance could be selected *in vitro*, we subjected Dd2 and 7G8 parasites to moderate levels of AZ (see “Experimental Procedures”) and selected resistant lines. These lines, termed AZ-R$_{DD2}$ and AZ-R$_{7G8}$, were assayed for their degree of AZ resistance in 96-h assays, in parallel with their parental lines Dd2 and 7G8. This produced AZ IC$_{50}$ values of 1.9 and 4.0 µM, respectively, as compared with IC$_{50}$ values of 124 and 228 nM, respectively, for the parental Dd2 and 7G8 lines, respectively, corresponding to highly significant (*p* < 0.001) 16- and 17-fold decreases in AZ susceptibility in the
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selected lines (Fig. 4; values reported in supplementary Table S3). The fact that resistant lines could be rapidly selected highlights the need to survey for resistance in AZ-containing antimalarial combination therapies.

As noted earlier, AZ and its progenitor erythromycin are chemically very closely related, and cross-resistance between the two antibiotics has been reported in several bacterial species including *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria gonorrhoeae* (56–59). Accordingly, we performed 96-h assays to investigate whether our *P. falciparum* AZ-resistant mutant lines would also be resistant to erythromycin. Indeed, this was observed but to a smaller extent, with significant (*p* < 0.05) 4- and 2-fold increases in erythromycin IC$_{50}$ values in AZ-R$_{Dd2}$ and AZ-R$_{7G8}$, respectively (Fig. 4).

We also screened for possible changes in parasite susceptibility to tetracycline, doxycycline, thiolestrogen, and CQ. Tetracycline and doxycycline were chosen because of their clinical use in combination with quinine, primarily for the treatment of uncomplicated, CQ-resistant *P. falciparum* malaria. As compared with its parental line 7G8, the mutant AZ-R$_{7G8}$ line displayed a significant (*p < 0.05*) decrease in tetracycline, doxycycline, and thiolestrogen IC$_{50}$ values, indicating that AZ-R$_{7G8}$ had become hypersensitive to these antimalarial drugs. No such change in susceptibility to these drugs was observed in the AZ-R$_{Dd2}$ line compared with Dd2. The CQ response was unchanged in both mutant AZ-resistant lines (Fig. 4).

To assess the stability of the AZ-resistant phenotype, both AZ-selected lines were maintained in the absence of drug pressure for 1 month and the AZ responses then retested. This revealed no alteration in IC$_{50}$ values as compared with parasites continuously cultured in the presence of AZ (IC$_{50}$ values for AZ-R$_{Dd2}$ and AZ-R$_{7G8}$ were 2.1 and 4.2 µM in the absence of drug pressure versus 1.9 and 4.0 µM in the presence of sustained drug pressure, respectively, in 96-h assays). These findings indicated that the selected AZ resistance phenotype was stable over time.

Identification of Mutations in the Apicoplast LSU rRNA and Pfrpl4 Genes in AZ-resistant *P. falciparum*—To examine the molecular basis of AZ resistance, we PCR amplified and sequenced the apicoplast LSU rRNA (the *P. falciparum* ortholog of bacterial 23 S rRNA) and Pfrpl4 genes as well as the nuclear Pfrpl22 gene (all are present as a single copy). We selected these potential targets based on their similarity with bacterial genes and published reports that mutations in these sequences can confer resistance to AZ and related macrolides in pathogenic bacteria (60–63).

In examining the *P. falciparum* apicoplast LSU rRNA (which bears 70% identity to the *E. coli* 23 S rRNA (60)), we first focused on A1875, which corresponds to the *E. coli* A2058 nucleotide in the peptidyltransferase center of domain V, and A706, which is equivalent to *E. coli* A754 in domain II. Mutations in these positions can confer macrolide resistance in bacterial species including *E. coli*, *Helicobacter pylori*, *S. pneumoniae*, and *Mycobacterium* (62). We also examined G1878 (which corresponds to *E. coli* G2061) as a mutation at this position can confer resistance to clindamycin and moderately decrease the susceptibility to AZ in *T. gondii* (64). These nucleotides remained unchanged in our AZ-resistant *P. falciparum* lines, as did the entire domains II (hairpin loop 35) and V that in
bacteria form a binding pocket for macrolides including AZ and that can also harbor other mutations conferring macrolide resistance (8, 12, 13, 65, 66).

Upon sequencing the full-length 2.7-kb apicoplast LSU rRNA gene, we identified a T to C mutation at nucleotide position 438 in the AZ-R7G8 line. No mutation was observed in this gene in AZ-RDd2. Sequence analysis confirmed that both the Dd2 and 7G8 parental lines carried the same T438 nucleotide, indicating that the AZ-R7G8 line had acquired this novel LSU rRNA U438C mutation during the period of AZ selection. Intriguingly, this mutation was found in region I, away from domains II and V that contain the majority of the mutations associated with AZ resistance (67).

During this study, we also identified a ribosomal rpl22 ortholog in P. falciparum (Pfrpl22; corresponding to PF14_0642 and originally annotated as encoding a hypothetical protein) based on the similarity of its encoded product with bacterial L22 proteins and an apicoplast-directed Rpl22 protein in T. gondii (see sequence alignment in supplemental Fig. 2). Sequence analysis of the full-length Pfrpl22 gene from the AZ-RDd2, AZ-R7G8, Dd2, and 7G8 parasite lines showed no sequence change.

Analysis of the Pfrpl4 gene, however, identified a single point mutation, resulting in a glycine (Gly, codon GGA) to valine (Val, codon GCA) substitution at codon 76 in both the AZ-RDd2 and AZ-R7G8 lines that was not present in either the parental Dd2 or 7G8 lines. This G76V mutation is present in the conserved P. falciparum motif 71VQKGLKAR79, which bears close similarity to the bacterial motif 61RQKGTGR69 (conserved residues underlined) present in the E. coli L4 protein (Table 1). The fact that both the Dd2 and 7G8 parasite lines were independently selected for AZ resistance and underwent the same mutation in their Pfrpl4 gene provides compelling evidence that this G76V mutation can contribute to P. falciparum resistance to AZ. For both the Pfrpl4 and LSU rRNA genes, the sequences were determined from PCR-amplified products and the electropherograms were unambiguous, implying that the mutant lines had a homogeneous population of mutant apicoplast genomes with no evidence of the wild-type sequences. At this time, we cannot exclude the possibility that undetected mutations occurred elsewhere in the apicoplast or nuclear genomes in our AZ-pressured lines.

Apicoplast-encoded Pfrpl4 Cannot Be Redirected to this Organelle from Episomally Replicating Transgene Plasmids—As an initial approach to assess whether the Pfrpl4 G76V mutation was sufficient for AZ resistance, we transfected mutant and wild-type Pfrpl4 alleles into several AZ-sensitive P. falciparum lines (3D7, Dd2, and GCO3). The native Pfrpl4 gene is located on the apicoplast genome, yet our transfection strategies were based on expression of this gene from plasmid DNA that was replicated in the nucleus. In an attempt to target recombinant Pfrpl4 to its host apicoplast organelle, these alleles were ligated to a DNA fragment encoding the complete apicoplast targeting sequence of ACP as this sequence was previously shown to direct trafficking of nuclear-encoded gene products to the apicoplast (35). This bipartite targeting sequence is composed of a SP and a transit peptide, and the apicoplast targeting sequence algorithm PlasmoAP (68) predicted the presence of a putative transit peptide sequence in Pfrpl4. Accordingly, we also generated constructs that fused just the SP sequence from the ACP gene to mutant or wild-type Pfrpl4. The recombinant ACP-Pfrpl4 and SP-Pfrpl4 mutant or wild-type gene fusions (with a V5 epitope tag or GFP at the 3' end) were cloned into expression plasmids under the control of calmodulin or hsp86 promoters, making a total of 16 constructs (69). Parasite lines transfected with sequence-verified plasmids expressing ACP-

| Organism          | Phenotype       | Amino acid sequence | Residues | Ref. |
|-------------------|-----------------|---------------------|----------|-----|
| P. falciparum     | Sensitive       | V Q K G L G K A R   | 71-79    |     |
| T. gondii         | Sensitive       | Q Q K G S G K A R   | 78-86    |     |
| D. radiodurans    | Sensitive       | G Q K G T G N A R   | 60-68    |     |
| S. pneumoniae     | Sensitive       | R Q K G T G R A R   | 66-74    |     |
| E. coli           | Sensitive       | R Q K G T G R A R   | 61-69    |     |

### Table 1

Alignment of the ribosomal protein L4 conserved motif from P. falciparum and other selected species

L4 mutations in AZ-resistant organisms are indicated as bold and boxed residues.
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Pfrpl4-GFP or ACP-Pfrpl4-V5 revealed no detectable protein expression (data not shown). Lines transfected with plasmids expressing SP-Pfrpl4-GFP fusions yielded cultures that expressed recombinant PfRpl4-GFP fusions. However, fluorescence microscopy revealed cytosolic localization of these fusions proteins (supplemental Fig. 1), indicating that the combined SP and Pfrpl4 putative transit peptide sequence were insufficient for apicoplast targeting. These experiments were repeated on several occasions, independent of which promoter (calmodulin or hsp86) was used to drive the transgene or whether the Pfrpl4 sequence was mutant or wild-type. We speculate that the inability to traffic Pfrpl4 to the apicoplast might reflect tight regulation of the apicoplast ribosomal translation machinery that is incompatible with Pfrpl4 overexpression in transfected parasites.

FIGURE 5. Structural models of the PFRpl4 wild-type (A) and G76V mutant (B) complexed with AZ. These models, derived using the D. radiodurans crystal structure of the L4 protein as a template (13), predict a steric clash between the side chain of Leu\textsuperscript{75} in the G76V mutant and AZ, consistent with this mutation conferring AZ resistance.

**DISCUSSION**

Our investigations into the antimalarial properties of AZ reveal this to be a slow-acting drug that becomes highly potent when extending parasite exposure from one to two generations, attaining IC\textsubscript{50} values as low as 50 nM (supplemental Table 2). AZ activity was not synergistic with CQ, amodiaquine, or artesunate. Furthermore, AZ potency was unaffected by point mutations in the DV transmembrane proteins PFCRT or PIP51.
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PiMDR1 that can reduce susceptibility to several antimalarials that act inside the DV, suggesting that this is not the primary site of action of AZ. Drug pressure studies resulted in the selection of AZ-resistant parasite lines that were found to harbor point mutations in the apicoplast-encoded *Pfrpl4* and LSU rRNA genes involved in apicoplast protein synthesis. These AZ-resistant parasites also displayed cross-resistance to erythromycin, a known inhibitor of the bacterial 50 S ribosomal subunit (56, 57, 70–73). Collectively, these data provide compelling evidence that the antimalarial properties of AZ are a result of its binding to the apicoplast 50 S ribosomal subunit and inhibiting protein synthesis in this organelle. These data complement separate studies directly implicating the apicoplast as the target of tetracycline and doxycycline in *P. falciparum* and clindamycin in *T. gondii* (64, 74).

In our study, AZ potency increased 30–230-fold upon prolonged exposure (from 48 to 96 h). These data are consistent with an earlier study (16) and provide a marked contrast to the comparator drugs thioistrepton and triclosan (that are both also thought to act in the apicoplast (41, 42, 44, 75)) and CQ (that acts in the DV (54)), which showed only minor gains in potency (Fig. 1). This “delayed death” phenotype was first described with *T. gondii* tachyzoites exposed to clindamycin and AZ (76). These antibiotics were found to not affect replication in the first generation of exposure, yet drastically reduced tachyzoite division in the second generation (64, 76). One proposed explanation for this phenotype was that these antibiotics render the parasites unable to establish a parasitophorous vacuole upon host cell reinvasion (28, 76). Alternatively, their effect on apicoplast protein synthesis might lead to insufficient levels of an as yet unidentified, apicoplast-encoded parasite factor required for the successful development of the progeny of drug-treated parasites (29, 74, 77).

Our *in vitro* studies reveal essentially additive effects between AZ and CQ, which is in contrast to earlier reports of *in vitro* synergy between these drugs with some *P. falciparum* lines (17). This discrepancy may be attributable to differences in methodology or analysis, or differences between the lines employed and for how long they had been adapted to *in vitro* culture. Our lack of synergy, reported with five lines tested on five separate occasions, provides evidence that these drugs have separate modes of action. AZ and CQ may nevertheless manifest some synergistic effects when used clinically. This is evidenced by a recent clinical trial in India in an area with a high prevalence of CQ-resistant malaria, which found that AZ–CQ combination therapy was significantly more efficacious in treating falciparum malaria than monotherapy with either agent (97% cure rates with AZ–CQ *versus* 33 and 27% with these individual agents, respectively (21)).

Direct evidence that AZ resistance in *P. falciparum* is mediated by changes in the bacterial-like apicoplast ribosome comes from our resistant mutant *in vitro* selection studies, which identified mutations in the apicoplast-encoded *Pfrpl4* and LSU rRNA genes. A key role for *Pfrpl4* is suggested by our finding that both the drug-pressured 7G8 and Dd2 lines underwent a G76V mutation in this gene. Similarly placed mutations in genes encoding L4 have been strongly implicated in macrolide resistance in several bacterial species (Table 1). For example, two studies observed G69C or G71R mutations in this gene in *S. pneumoniae* strains selected for AZ resistance (56, 57). These mutations correspond to residues 74 and 76 in *Pfrpl4* (Table 1). Other AZ-resistant *S. pneumoniae* strains also show insertions, deletions, or multiple mutations in this L4 conserved site (71, 73). Similar L4 mutations have also been identified in macrolide-resistant strains of *Streptococcus pyogenes*, *H. influenzae*, *Staphylococcus aureus*, and *E. coli* (70, 78, 79). Whereas many of those studies only reported an association, genetic complementation studies have provided convincing evidence that L4 mutations can confer AZ resistance. For example, genetic transformation of the AZ-sensitive *S. pneumoniae* R6 strain with the mutant *rpl4* alleles G69C, 67QKG69 to QSQKG, 67GTG71 to TPS, 67QKG71 to QT, and 64PWRQ67 to PQ produced 8–15, 60–16, and 16-fold decreases, respectively, in the susceptibility of these transformed strains to AZ (56, 80–82). In the converse approach, complementation of erythromycin-resistant *E. coli* mutants with plasmids expressing wild-type *rpl4* or *rpl22* genes resulted in decreased resistance to erythromycin (70). Our study also adopted a complementation approach by trying to express the apicoplast-encoded *Pfrpl4* gene (both wild and mutant types) in different AZ-sensitive parasite strains. However, despite multiple and varied attempts, we could not obtain recombinant parasite lines that targeted recombinant *Pfrpl4* to the apicoplast. An alternative and more definitive approach would be to perform allelic exchange of the endogenous *Pfrpl4* gene with a mutant *Pfrpl4* sequence that is being replicated inside the apicoplast. This experiment can only be undertaken once there is a suitable selectable marker for transfection of the *P. falciparum* apicoplast.

We note that one of our AZ-resistant lines, AZ-R7G8, also harbored a LSU rRNA mutation (U438C) in domain I. This is away from domains II and V that form part of the macrolide binding pocket in the bacterial 50 S ribosomal subunit and that house the majority of the 23 S rRNA mutations associated with AZ resistance (67). Nonetheless, recent reports document the presence of 23 S rRNA domain I mutations (A138G, A260G, A373T, T389C, or A449C) in *S. pneumoniae* isolates (83, 84). This might contribute to the fact that our AZ-R7G8 mutant had a higher AZ Cmin value than AZ-RDd2. The AZ-R7G8 line also displayed significantly increased *in vitro* sensitivity to thioistrepton, tetracycline, and doxycycline. Thioistrepton, a peptide antibiotic, inhibits protein translation by blocking GTPase activity in the 23 S rRNA domain II (85), and studies have shown that an A1067U mutation (*E. coli* numbering) can confer thioistrepton resistance in *E. coli* and *P. falciparum* (42, 86). Tetracycline and doxycycline block protein synthesis by binding to the 30 S subunit that inhibits attachment of tRNA to the A-site of ribosomes (87, 88). Photolysis experiments performed with radiolabeled tetracycline and *E. coli* ribosomes, however, provide evidence that this antibiotic can also interact with nucleotides in the central loop of the 23 S RNA domain V (89). Domains II and V, however, were invariant in AZ-R7G8, suggesting that the LSU rRNA domain I mutation we observed in this line was responsible for the observed hypersensitivity to thioistrepton, tetracycline, and doxycycline. A possible explanation is provided by structural studies. Analysis of the structure of the large ribosomal subunit
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from Haloarcula marismortui complexed with its repertoire of ribosomal proteins showed that the three-dimensional catalytic structure of the 23 S rRNA is maintained by interactions of all six domain subunits with each other through extensive hydrogen bonding and other non-covalent interactions (67). Cryo-electron microscopy studies have also shown that mutant L4 proteins can exert long-range structural effects on the 30 S subunit in erythromycin-resistant E. coli ribosomes (10). We posit that the hypersensitivity of AZ-R G to thiostrepton, tetracycline, and doxycycline might be due to the LSU rRNA domain I U438C mutation causing structural changes that are transmitted to the antibiotic binding sites on the 50 S and 30 S rRNA subunits. We also note that this increased antibiotic sensitivity, if found to occur frequently in AZ-resistant parasites, would promote the potential use of these antibiotics as partner drugs for combination therapy. Our findings of mutations in pfrpl4 and the LSU rRNA are consistent with published data from pathogenic bacteria (including S. pneumoniae and H. influenzae) that directly implicate mutations in the 23 S rRNA or the genes encoding ribosomal proteins L4 and L22 as causal determinants of macrolide resistance (62, 79, 90). Furthermore, our finding that resistance to AZ could be rapidly selected in vitro is an important consideration in the testing of future AZ-containing antimalarial combination therapies.

Other reported mechanisms of macrolide resistance in bacteria are methylation of the A2058 nucleotide in the 23 S rRNA domain V by a methyltransferase encoded by the erm (erythromycin ribosome methylation) genes, and drug efflux mediated by an efflux pump encoded by the mef (macrolide efflux) genes (62, 92). Methylation of this critical nucleotide acts to reduce macrolide binding to the ribosome, and tends to predominate over point mutations as the major mechanism of macrolide resistance in bacterial species such as Streptococcus and Staphylococcus that harbor multiple rRNA operons. Our analysis of the recently released P. falciparum (PB3 strain) apicoplast genome (GenBank accession number DQ642846, sequenced by the Broad Institute, Cambridge, MA) reveals that this 29-kb genome has only one functional copy of the LSU and small subunit rRNA genes. This resolves earlier questions about the possible presence of a second LSU gene in the apicoplast (61). Our analysis also shows the absence of any putative apicoplast gene coding for an adenosine methyltransferase or an active drug efflux pump. Thus, at this time it would appear less likely that rRNA methylation or drug efflux mechanisms could operate to confer AZ resistance in P. falciparum.

L4 and L22 are the early assembly proteins of the 50 S ribosomal subunit and play a pivotal role in scaffolding the ribosomal domains (10). The crystal structure of L4 from Thermotoga maritima defines a loop of 55 residues that displays the highest degree of phylogenetic conservation across the L4 sequences (corresponding in Pfrpl4 to residues 47–101) and that represents the main rRNA binding site (93). RNA-protein cross-linking studies in E. coli have shown that this conserved L4 loop interacts with 23 S rRNA nucleotides 284–350 and 580–670 in domains I and II, respectively (94). Cryo-electron microscopy of erythromycin-resistant E. coli ribosomes indicates that sequence changes in L4 or L22 can alter the spatial organization of nucleotides in 23 S rRNA domains II, III, and V, and the conformation of the C-terminal ends of these proteins that form a segment of the polypeptide exit tunnel. These structural rearrangements can significantly change the diameter of the peptide tunnel, affecting macrolide accessibility to the peptidyltransferase center in domain V and preventing the macrolide from inhibiting protein synthesis and egress from the peptide tunnel (10, 91). Structural analysis of the entire 50 S ribosomal subunit of H. marismortui containing L4 and L22 has localized binding of AZ and its progenitor erythromycin to a hydrophobic drug-binding pocket formed by critical 23 S nucleotides A2058, A2059, and C2611 (E. coli numbering). One key interaction is the formation of a hydrogen bond between the desosamine sugar at position C-5 of the macrolide lactone ring and A2058 (65). A similar pattern of interactions between AZ and these rRNA nucleotides was also identified in crystallography studies of the D. radiodurans large subunit ribosome. This work also identified an additional binding site for AZ, formed by ribosomal proteins L4 and L22 and domain II of the 23 S rRNA (13). AZ was observed to directly interact with ribosomal proteins L4 (notably with the Tyr59, Gly60, Gly63, and Thr64 residues) and L22 (the Arg111 residue) through hydrogen bonding and hydrophobic interactions, respectively (13).

Our modeling of wild-type and mutant Pfrpl4, based on structural data from D. radiodurans that identified AZ binding to L4 (13), predicts a dramatic impact of the G76V mutation on the neighboring Leu75 residue as a result of its reorientation in the mutant protein. This leads us to propose that the reoriented Leu75 residue can create a steric clash with AZ, resulting in reduced binding. An alternative explanation is that this G76V mutation prevents AZ from binding to the nascent polypeptide exit tunnel in the 50 S ribosome by constricting the macrolide-binding site. In either event, this study provides compelling evidence that AZ acts upon the P. falciparum apicoplast and highlights the importance of screening for possible mutations in the LSU rRNA, Pfrpl4 and Pfrpl22 genes in ongoing clinical trials designed to assess the efficacy of AZ-containing antimalarial combination therapies.

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