Telithromycin and Quinupristin-Dalfopristin Induce Delayed Death in *Plasmodium falciparum*[▽]

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Antibacterial agents are used in malaria therapy due to their effect on two prokaryote organelles, the mitochondrion and the apicoplast. We demonstrate here that the ribosome-blocking antibiotics telithromycin and quinupristin-dalfopristin, but not linezolid, inhibit the growth of *Plasmodium falciparum*. Both drugs induce delayed death in the parasite, suggesting that their effect involves the impairment of apicoplast translation processes.

The tropical disease malaria is a major health threat, as well as a great economic burden, and chemotherapeutic measures are increasingly encountering parasite drug resistance. To circumvent this problem, antibiotics like doxycycline or clindamycin are increasingly being used in combination therapy together with classical malaria medications like quinine or mefloquine (26). The antimalarial effect of antibiotics, which target typical prokaryotic structures, can be explained by the presence of two parasite organelles of prokaryotic origin, the mitochondrion and the apicoplast.

The apicoplast, which was first described in *Plasmodium* a decade ago (17, 33), likely arose through a process of secondary endosymbiosis (14). Although the full extent of its function is unknown, it appears to be essential for the anabolic synthesis of fatty acids, isoprenoids, and heme. The circular 35-kb apicoplast genome encodes only 30 proteins, and thus, the apicoplast proteome is supplemented by as many as 500 nucleus-encoded, apicoplast-targeted proteins, which resemble 10% of the predicted parasite proteome and which are trafficked into the organelle. The apicoplast is essential for parasite survival (9, 21, 28, 31), and its elimination by select chemotherapeutics results in a slow killing of the parasite, which has consequently been termed “delayed death” (6–8, 10, 24, 34, 35).

The delayed death effect in malaria parasites is defined by slow growth inhibition after 48 h of treatment with drug concentrations that are more than 10-fold higher than those needed to inhibit 50% of parasite growth following a further asexual cycle (10). A delayed death effect is typical for antibacterials that inhibit prokaryote translation, like azithromycin, clindamycin, or doxycycline (4, 6, 7, 10, 14).

Several new antibiotics are currently in clinical use to particularly combat infections of gram-positive bacteria like *Staphylococcus aureus*, *Streptococcus pneumoniae*, or *Enterococcus faecium* (1). These include the streptogramin combination drug quinupristin-dalfopristin, the ketolide telithromycin, and the oxazolidinone linezolid. All three drugs target the

bacterial ribosome (1, 5), and a possible activity on the apicoplast protein synthesis of the malaria parasite could therefore be anticipated. Antibiotics are often used to treat bacterial coinfections in patients suffering from malaria, and the use of antibiotics with an antiplasmodial effect would therefore represent an additional advantage in malaria therapy. Coherently, the aims of this study were to investigate the antimalarial effects of quinupristin-dalfopristin, telithromycin, and linezolid and to determine their possible cellular targets.

The compounds were screened on the human malaria pathogen *Plasmodium falciparum* at concentrations between 1 nM and 100 μ M. Synchronized ring stages of *P. falciparum* strain 3D7 were plated in 96-well-plates at a parasitemia of 1% in the presence of azithromycin, quinupristin-dalfopristin, or telithromycin (dissolved in dimethyl sulfoxide [DMSO]) or of doxycycline or linezolid (dissolved in water). Incubation of parasites with DMSO alone at a concentration of 0.5% by volume was used as a negative control. The parasites were cultivated in vitro as previously described (12), with a medium change at day 3 of the assay. Four incubation periods, 48 h, 72 h, 96 h, and 120 h, were investigated. The viability of the parasites was screened subsequently by using the Malstat assay, which measures the activity of the *Plasmodium*-specific enzyme lactate dehydrogenase (CAS: 9001-60-9), as previously described (11, 15, 16).

Malstat screening revealed an inhibition of parasite growth in cultures that were incubated with either quinupristin-dalfopristin or telithromycin; however, no inhibition was detected when parasites were cultured in the presence of linezolid at any of the time points investigated (Table 1). Treatment with doxycycline and azithromycin, which were used as positive controls, resulted in parasite growth inhibition with a 10-fold decrease in the 50% inhibitory concentrations (IC₅₀s) after 96 h compared to 48 h of incubation time (Table 1). These data reflect the typical delayed death effect previously described for these antibiotics (6, 7, 27). A similar delayed death effect was observed in parasites treated with quinupristin-dalfopristin or telithromycin, which was also reflected in a more-than-10-fold decrease in the IC₅₀s of both antibiotics during the second replication cycle (Table 1). No inhibition of parasite growth was detected in cultures treated with linezolid (Table 1).

Doxycycline and azithromycin were previously reported to

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TABLE 1. Antimalarial activities of the antibiotics under study

Time (h)	Mean IC ₅₀ (μ M) $\pm$ SEM				
	Doxycycline	Azithromycin	Quinupristin-dalfopristin	Telithromycin	Linezolid
48	5.42 $\pm$ 1.650	9.04 $\pm$ 0.709	6.56 $\pm$ 1.475	8.08 $\pm$ 2.590	NI ^a
72	1.49 $\pm$ 0.492	4.57 $\pm$ 1.240	2.61 $\pm$ 0.789	1.04 $\pm$ 0.045	NI
96	0.72 $\pm$ 0.051	0.94 $\pm$ 0.163	0.55 $\pm$ 0.411	0.28 $\pm$ 0.146	NI
120	0.12 $\pm$ 0.037	0.41 $\pm$ 0.175	0.07 $\pm$ 0.028	0.44 $\pm$ 0.381	NI

^a NI, no inhibition.

promote developmental arrest of *P. falciparum* during the second replication cycle, where parasites initiated schizogony but failed to form mature schizonts (6, 7, 27). To investigate if a similar developmental halt occurs in parasites treated with quinupristin-dalfopristin or telithromycin, Giemsa smears of cultures that were incubated with the antibiotics at their IC₅₀ (96 h)s were prepared. The developmental stages were then counted at the four time points of investigation. In parasites treated with telithromycin and particularly in those treated with quinupristin-dalfopristin, a delay of about 5 to 10 h in the first replication cycle was observed (Fig. 1A). The parasites were subsequently arrested in the second replication cycle. While developmental arrest for doxycycline-treated and azithromycin-treated parasites was mostly observed in the transition from early to mature schizont stages (Fig. 1A), parasites treated with either quinupristin-dalfopristin or telithromycin stopped their development predominantly in the transition from the late trophozoite stage to the early schizont stage (Fig. 1A).

The exact stage of arrest was further investigated by transmission electron microscopy. Parasite cultures were again incubated with the antibiotics at their IC₅₀ (96 h)s and then collected at 96 h of drug treatment. The specimens were fixed in 1% glutaraldehyde and 4% paraformaldehyde in phosphate-buffered saline and processed for electron microscopy as described previously (20). While untreated parasites had either formed mature rupturing schizonts (Fig. 1B) or developed to ring stages (data not shown), drug-treated parasites had stopped their development at the schizont stages and exhibited abnormal morphologies. For doxycycline-treated and azithromycin-treated parasites, an arrest was mostly observed in schizonts that had formed multiple nuclei. Telithromycin-treated parasites, on the other hand, were predominantly arrested at a stage where only two nuclei are present (Fig. 1B). Schizonts treated with any of the four drugs usually showed vacuolization. This was particularly distinctive after treatment with quinupristin-dalfopristin, where no nucleus division was distinguishable in the majority of the arrested parasites (Fig. 1B).

The antimalarial effect of antibiotics was described for the first time more than 50 years ago (13). Their mode of action has been attributed to interference with the parasite mitochondrion and apicoplast, but only recently have the immediate and delayed death effects of selected antibacterials been studied in more detail. The antibiotic thiostrepton was reported to target the apicoplast and kill parasites immediately by blocking trophozoite development during the first asexual cycle (10). A delayed death effect, on the other hand, was reported for antibiotics, like azithromycin, clindamycin, or the tetracyclines, that interfere with the apicoplast (4, 6, 7, 10, 27). Here, inhi-

bition of the apicoplast translation machinery results in the distribution of nonfunctional apicoplasts into daughter merozoites, arresting the schizont during maturation (7). The apicoplast is proposed to be involved in the formation of daughter cell plasma membranes via type II fatty acid synthesis, which would only be effective in the second cycle (7). Clindamycin is also assumed to target the apicoplast, leading to failed cytokinesis during the second replication cycle (10). The delayed death effect of clindamycin was additionally demonstrated in vivo in adult patients, with a decline of asexual parasite numbers to nearly undetectable levels after 72 h of treatment (4). Contradictory results were presented for the antibiotics rifampin and ciprofloxacin, though, which were reported to either cause delayed death or kill the parasite immediately (6, 7, 10, 22).

A delayed death effect has recently also been assigned to a nonantibiotic inhibitor of the apicoplast, deoxyspergualin (23). Deoxyspergualin inhibits trafficking of nucleus-encoded, apicoplast-targeted proteins during the first asexual cycle, which leads to apicoplast missegregation and subsequent impairment in the second asexual cycle. Other apicoplast-targeted compounds, like selected inhibitors of the mevalonate-independent isoprenoid pathway, as well as of type II fatty acid and heme biosynthesis, cause immediate killing of the parasite (22, 31).

We show here that two antibiotics, telithromycin and quinupristin-dalfopristin, trigger delayed death in the malaria pathogen. The drug concentrations necessary for the half-maximal inhibition of parasite growth in vitro (quinupristin-dalfopristin, IC₅₀ (48 h) = 5.18 mg/liter and IC₅₀ (96 h) = 0.43 mg/liter; telithromycin, IC₅₀ (48 h) = 6.56 mg/liter and IC₅₀ (96 h) = 0.23 mg/liter) lie within the range of previously published therapeutically obtained pharmacokinetics for the two antibiotics. The mean maximal concentration in plasma had previously been determined to be 8.65 mg/liter for quinupristin-dalfopristin and 1.9 mg/liter for telithromycin after the application of 12 mg/kg via a 1-h infusion (3) or a single oral dose (19).

Linezolid was devoid of antiplasmodial activity. The antibiotic binds to the A site of the 70S ribosome, with its binding site to a large extent overlapping that of the antimalarially effective antibiotic chloramphenicol and to a lesser extent that of lincosamines (32). The lack of activity of linezolid may be related to subtle differences in ribosome structure between the parasite organelles and those of pathogenic bacteria (18), thereby altering its binding affinity. Another possible explanation for the inactivity of linezolid might be an inability to pass the apicoplast's membranes.

Due to the developmental arrest of treated parasites in the schizont stage of the second replication cycle, we propose that telithromycin and quinupristin-dalfopristin target the apico-

FIG. 1. Antibiotic treatment causes developmental arrest and morphological abnormalities during the second replication cycle. (A) Histogram indicating parasite stages during drug treatment after different incubation times compared to an untreated control. Synchronized parasites were incubated with the antibiotics at the respective IC_{50} s or with 0.5% DMSO by volume (untreated control) over three life cycles. Giemsa smears were made at different time points, and the ring stages, trophozoites, early schizonts, and mature schizonts were counted. (B) Transmission electron micrographs revealing antibiotic-induced ultrastructural abnormalities after developmental arrest at 96 h of assay. HZ, hemozoin; M, merozoite; N, nucleus; V, vacuole. Bar, 1 μ m.

plast. The two antibiotics additionally induce a delay in parasite maturation in the first replication cycle and then arrest the parasite at an earlier stage of the second cycle, namely, at the transition from the late trophozoite stage to the early schizont stage. This might be due to an additional translational inhibi-

tion in the mitochondrion, as has been attributed to the antibiotics rifampin and tetracycline (22). Both organelles are intimately linked in *Plasmodium*, in physical terms as well as regarding components of housekeeping and heme biosynthesis (2, 25, 29, 30).

The inclusion of antiparasitic antibiotics in combination therapy offers an unprecedented opportunity to combat malaria and oppose the spread of drug resistance. Thus, the continued exploration of existing antibiotics for their possible antiparasitic activity is a prerequisite for effective control measures. Our results indicate that telithromycin and quinupristin-dalfopristin inhibit the growth of *P. falciparum* at clinically relevant concentrations and therefore may represent promising new candidates for malaria combination therapy.

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