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Borrelidin, a potent antimalarial: stage-specific inhibition profile of synchronized cultures of *Plasmodium falciparum*

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Malaria has been historically recognized as one of the world's most devastating diseases. It has recently been attracting increased global attention and has been the focus of intensified efforts to combat it. Globally coordinated interventions are proving highly successful, such that there is now a pressing and increasing need to prevent, treat or, where possible, eliminate the disease (http://www.who.int/malaria/en/). Malaria is endemic throughout much of the tropics and has traditionally caused more than 250 million cases and nearly 1 million deaths annually. *Plasmodium falciparum* malaria is especially destructive and can cause death relatively quickly if cerebral malaria develops. Generally, most of those who die from malaria are children under 5 years old. Emergence of parasite strains resistant to commonly used curative clinical drugs, such as chloroquine, mefloquine, quinine, sulfadoxine/pyrimethamine and halofantrin, is a serious and widespread problem, preventing effective treatment and hindering disease elimination efforts. Under such circumstances, newly developed artemisinin derivatives have become the most effective anti-malarial drugs, and the World Health Organization now recommends artemisinin-based combination therapies for malaria treatment. However, artemisinin-based combination therapies is not a panacea for all cases of malaria, because safety of the artemisinin with regard to use during first trimester pregnancy is yet to be established[1] and resistance to artemisinin derivatives has already been found along the Thai-Cambodian border.[2] Therefore, inexpensive and potent antimalarial drugs, especially those that have different modes of, are urgently required—and will be on a continuing basis because of the ability of the parasites to quickly develop drug resistance.

We have been screening microbial metabolites to discover antimalarial compounds using drug-resistant *Plasmodium* parasites.[3–8] In the course of our screening, we found borrelidin, produced by an actinomycete strain OM-0060, that proved to be more potent than chloroquine or artemether (using *in vivo* oral administration) against drug-resistant *P. yoelii*.[6] The reported biological activities of borrelidin are suggested to be the results of protein synthesis inhibition, caused by the effect on threonyl-tRNA synthetase.[9] Borrelidin is also known to inhibit yeast (*Saccharomyces cerevisiae*) cyclin-dependent kinase (CDK), where the target is cdc28/cln2 complex formation.[10] It has also been reported that borrelidin induces apoptosis in HUVEC.[11] However, the mechanism of antimalarial activity of borrelidin has not yet been identified. In this paper, we report the stage-specific action of borrelidin *in vitro,* using a drug-resistant parasite strain, determined by parasite lactate dehydrogenase (p-LDH) activity[3] and by morphological observation. Furthermore, we tested the effect of L-threonine, which appears to curtail the antimalarial activity of borrelidin, possibly through its effect on threonyl-tRNA synthetase, with a view to gaining an initial assessment of the antimalarial mode of action.

The drug-resistant *P. falciparum* K1 strain was used to produce synchronous parasite cultures, as previously described.[12] Erythrocytic replication of *P. falciparum* occurred through a 48-h cycle with morphological changes. For experimental purposes, parasite stages were determined as follows; ring stage (0–12 h after the last synchronization), which has a ring form; early-trophozoite stage (12–24 h after the last synchronization), which has a large or thick ring appearance with no pigment; trophozoite stage (24–36 h after final synchronization), which has an even cytosolic component and pigment with a single nucleus; schizont stage (36–42 h after final synchronization), which has two or more nuclei; Mature schizont stage (44–47 h after final synchronization), which exhibits a post-mitotic form. Using a 12-well culture plate, the drugs (50 μl) dissolved in 25% EtOH (borrelidin) or 5% dimethyl sulfoxide (DMSO) (arte-

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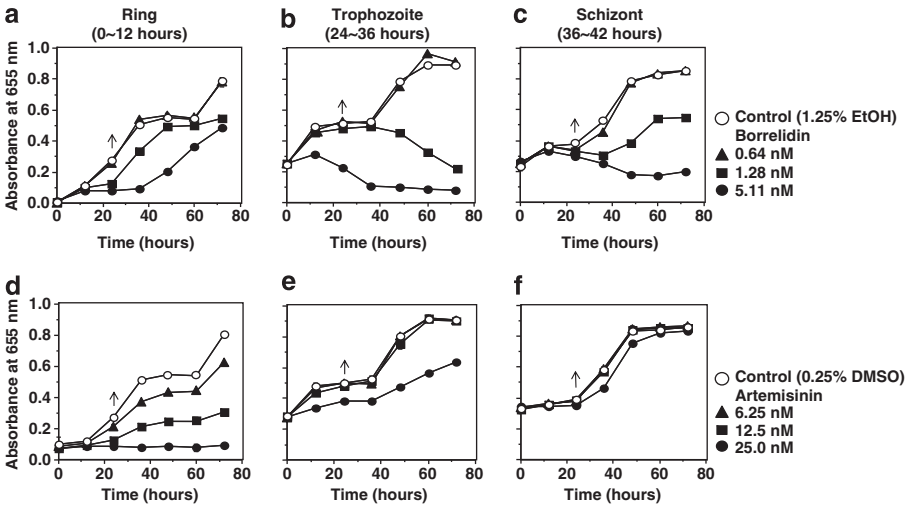

Figure 1 Time course of p-LHD activity in different stages cultured with various concentrations of borrelidin or artemisinin. (**a**, **d**), (**b**, **e**) and (**c**, **f**) show ring stage, trophozoite stage and schizont stage, respectively. Arrows indicate the point of drug removal.

misinin) or solvents alone were added to 950 µl of cultures at 0, 24, or 36 h after the last synchronization (that is, at the initiation of ring, trophozoite and schizont stage culture, respectively), followed by incubation for 24 h. After incubation, the parasite cultures were individually collected and washed three times with complete medium. Then parasites were transferred to a new 12-well culture plate and incubated for 48 h. During the process, 80 µl of each culture was harvested every 12 h for p-LDH assay. Blood smears were made every 24 h and observed under a light microscope. Test drug concentration was determined by IC_{50} values (borrelidin and artemisinin are 1.9 and 18 nM, respectively), using asynchronous cultured parasites. To explore the possibility of threonyl-tRNA synthetase inhibition of borrelidin, we assayed the effect of antimalarial activity of borrelidin in the presence of excessive quantities of L-threonine (1 or 5 mM), using the antimalarial assay described previously.[3] Briefly, asynchronous culture of the *P. falciparum* K1 strain was incubated with the drug for 72 h and a p-LDH assay was used for antimalarial evaluation.

Figure 1 shows the time course of p-LDH activity in different parasite stages treated with test drugs. The p-LDH activity in controls (1.25% EtOH or 0.25% DMSO) increased stepwise and showed normal development (Figures 1 and 2A) confirming that the solvents did not influence the parasite culture. The p-LDH activity of all stages was not affected by 0.64 nM borrelidin (Figures 1a–c). When exposed to 1.28 and 5.11 nM borrelidin, the p-LDH activity of ring-stage parasites increased after removing the drug (Figure 1a). In contrast, the p-LDH activity of trophozoite stage parasites exposed to 1.28 and 5.11 nM borrelidin decreased remarkably after removing the drug (Figure 1b). The p-LDH activity of schizont stage parasites exposed to 5.11 nM borrelidin also decreased, although p-LDH of the parasites exposed to 1.28 nM borrelidin increased after removing the drug (Figure 1c). The p-LDH activity of the ring-stage parasites exposed to 25 nM artemisinin was suppressed throughout the entire culture period, even though the drug was removed, and this occurred in a dose-dependent manner (Figure 1d). In the trophozoite and schizont stage, p-LDH activity was not suppressed or decreased (Figures 1e and f). These observations indicate that borrelidin has a specific inhibitory effect on the trophozoite stage parasites and it acts differently to artemisinin.

Figure 2 shows morphology of parasites. Control parasites using 0.25% DMSO showed normal development (Figure 2A), 1.25% EtOH being identical to 0.25% DMSO (data not shown). Ring-stage parasites exposed to borrelidin 5.11 and 1.28 nM showed developmental retardation compared with control parasites (Figures 2B and E). Parasites gradually shrunk when 5.11 nM borrelidin was added at the trophozoite stage (Figure 2C). With 1.28 nM borrelidin exposure to trophozoite-stage parasites, schizont and merozoite forms exhibited developmental delay and a few new ring parasites were observed at 96 h (Figure 2F). When parasites were exposed to 5.11 nM borrelidin at the schizont stage, mature schizont forms were observed, but new ring-form parasites did not appear (Figure 2D), while 1.28 nM borrelidin caused only slight retardation in development of schizont parasites (Figure 2G). Ring-stage parasites exposed to artemisinin (25 nM) shrunk gradually (Figure 2H), whereas trophozoite-stage parasites exposed to artemisinin showed delayed development (data not shown) and schizont-stage parasites exposed to artemisinin were little affected (data not shown).

Figure 3a shows the effect of L-threonine on the antimalarial activity of borrelidin. The antimalarial activity of borrelidin was significantly reduced in the presence of 1 or 5 mM of L-threonine. In contrast, the antimalarial activity of artemisinin was not affected (Figure 3b).

Borrelidin has been reported to have several biological activities, most of which are believed to be due to protein synthesis disruption caused by the inhibition of threonyl-tRNA synthetase.[9,13] In this experiment, borrelidin inhibited the trophozoite-stage parasites the most, which corresponds with the time of RNA synthesis and protein synthesis.[14] Therefore, the compound might inhibit *P. falciparum* threonyl-tRNA synthetase. Ruan *et al.*[15] reported borrelidin is tight binding inhibitor of *E. coli* threonyl-tRNA synthetase and the amino acid sequence of the binding site is found in the enzyme in humans, *S. cerevisiae* and *P. falciparum*. As the expression of this enzyme is regulated by a feedback mechanism,[13] we tested the effect of threonine on parasite growth, by reference to Kawamura *et al.*[11] and found that the antimalarial activity of borrelidin was curtailed by threonine (Figure 3a). Therefore, borrelidin might bind threonyl-tRNA synthetase, as growth inhibition and retardation was observed after drug removal, especially in trophozoite-stage parasites. Borrelidin is also known to inhibit yeast (*S. cerevisiae*) CDK, where the target is cdc28/cln2 complex formation.[10] *P. falciparum* CDKs have been isolated and identified.[16–18] Among them, three kinds of *P. falciparum* CDKs (PfPK5, PfPK6 and Pfmrk) are suggested as drug targets.[19] Cdc28

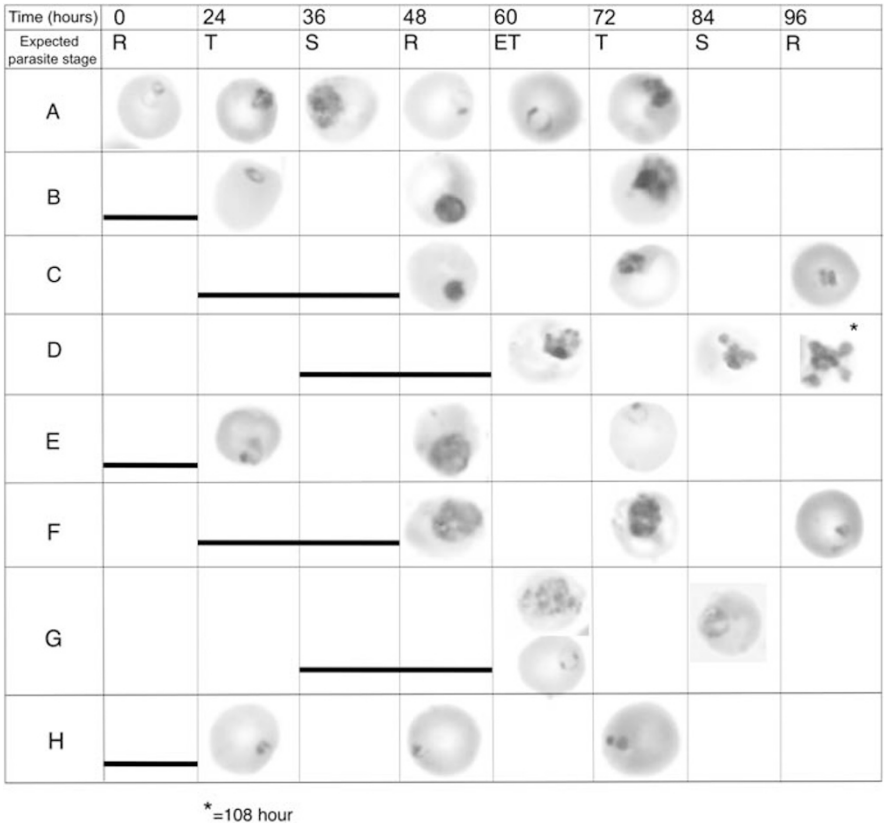

Figure 2 Morphological observation of parasites exposed to 5.11 or 1.28 nM borrelidin and 25 nM artemisinin. EtOH (1.25%) or DMSO (0.25%) was used as controls. (A) Control (DMSO) parasites; (B) borrelidin (5.11 nM) treated at ring stage; (C) borrelidin (5.11 nM) treated at trophozoite stage; (D) borrelidin (5.11 nM) treated at schizont stage; (E) borrelidin (1.28 nM) treated at ring stage; (F) borrelidin (1.28 nM) treated at trophozoite stage; (G) borrelidin (1.28 nM) treated at schizont stage; (H) artemisinin (25 nM) treated at ring stage. R, ring stage; ET, early trophozoite stage; T, trophozoite stage; S, schizont stage. Asterisk denotes 108-h parasite. Black bar indicates drug exposure period.

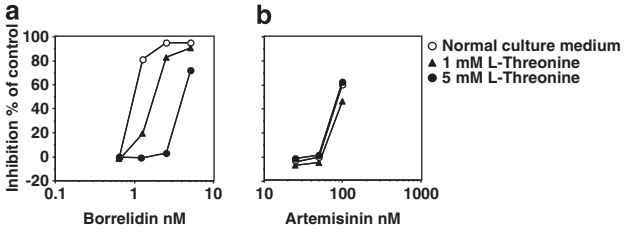

Figure 3 Antimalarial activity of borrelidin and artemisinin in the presence of excessive quantities of L-threonine. (**a**) Dose-dependent antimalarial activity of borrelidin; (**b**) dose-dependent antimalarial activity of artemisinin. L-Threonine was added to antimalarial assay culture medium at a concentration of 1 or 5 mM.

has homology to cdk1 (human CDK) and PfPK5 has homology to cdk1 and cdk5 (human CDK). This suggests that borrelidin may have an inhibitory impact on *P. falciparum* CDKs. PfCDKs are expressed stage specifically. PfPK5 is expressed at transcription level and protein level in the schizont stage and it has kinase activity, responsible for nuclear division of schizont-stage parasites.[16] In our study, borrelidin did not disrupt division of nuclei, but did affect parasite development in the schizont stage. Thus, the effect of borrelidin on the schizont stage may not involve PfCDKs inhibition. Unfortunately, we were not able to determine the relationship between borrelidin and apoptosis in *P. falciparum*. The apoptosis mechanism of *P. falciparum* in erythrocytic stages is not fully elucidated, although there are some reports of apoptosis using existing drugs, such a chloroquine and staurosporine.[20–23]

Recently, it was reported that macrolide antibiotics, such as azithromycin, caused the 'delayed death' of *P. falciparum* and their target was the apicoplast.[24–26] Although borrelidin is also a macrolide, its mode of action may well be different because borrelidin did not show 'delayed death' in this experiment.

In this study, we examined the stage-specific bioactivity of borrelidin, monitored by p-LDH activity and morphology, and carried out a preliminary analysis of its mode of action on *Plasmodium* parasites. We found that borrelidin showed stage-specific growth inhibition, most strongly in the trophozoite stage. We also observed that borrelidin does not appear to inhibit nuclear division when exposed at schizont stage. These results indicate that borrelidin acts differently than artemisinin and so the compound may prove to be an interesting candidate for further research to facilitate the production of new antimalarials with novel modes of action.

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