Analogs of natural aminoacyl-tRNA synthetase inhibitors clear malaria in vivo

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Malaria remains a major global health problem. Emerging resistance to existing antimalarial drugs drives the search for new antimalarials, and protein translation is a promising pathway to target. Here we explore the potential of the aminoacyl-tRNA synthetase (ARS) family as a source of antimalarial drug targets. First, a battery of known and novel ARS inhibitors was tested against *Plasmodium falciparum* cultures, and their activities were compared. Borrelidin, a natural inhibitor of threonyl-tRNA synthetase (ThrRS), stands out for its potent antimalarial effect. However, it also inhibits human ThrRS and is highly toxic to human cells. To circumvent this problem, we tested a library of bioengineered and semisynthetic borrelidin analogs for their antimalarial activity and toxicity. We found that some analogs effectively lose their toxicity against human cells while retaining a potent antiparasitic activity both in vitro and in vivo and cleared malaria from *Plasmodium yoelii*-infected mice, resulting in 100% mice survival rates. Our work identifies borrelidin analogs as potent, selective, and unexplored scaffolds that efficiently clear malaria both in vitro and in vivo.

aminoacyl-tRNA synthetase | malaria | drug design | borrelidin | plasmodium

With ~200 million clinical cases and more than 600,000 attributed deaths per year (World Health Organization Malaria Report 2013), malaria is one of the most severe infectious diseases for which there is no effective vaccine. Most deaths by malaria are caused by *Plasmodium falciparum*, one of the five distinct *Plasmodium* species that infect humans, whereas the high morbidity of *Plasmodium vivax*, which can cause dormant liver stage infections, also contributes to the enormous economic burden of the disease. Because malaria parasites contain an essential organelle of bacterial origin, the apicoplast, numerous antibacterials kill malaria parasites (1–4) and are commonly used in malaria prophylaxis or as components of multiple drug therapies (5, 6). However, the emergence of multiresistant parasites compromises the efficacy of many existing chemotherapies, leading to a growing urgency for the search of new antimalarials (7–11).

The protein synthesis machinery is an excellent target for the development of new anti-infectives. Indeed, commonly used antimalarial compounds such as doxycycline inhibit *Plasmodium* ribosomal function. Among the less exploited enzymes of the translation machinery is the family of aminoacyl-tRNA synthetases (ARS). These ancestral enzymes catalyze the correct attachment of amino acids to their cognate tRNAs and thus are responsible for the correct establishment of the genetic code. An important example of the clinical application of an ARS inhibitor is provided by the antibiotic mupirocin (pseudomonic acid; marketed as Bactroban, GlaxoSmithKline), which selectively inhibits bacterial isoleucyl-tRNA synthetase without inhibiting its human homolog. Although proven antibacterial drug targets (12–15), these enzymes have only recently been highlighted as antimalarial drug targets (16–18).

A major limitation of most antimalarial drugs is their inability to affect the liver stages of malaria, including *P. vivax* and *P. ovale* hypnozoites. The essential role of ARS in both liver and blood stages of malaria represents an additional advantage for their use as antimalarial targets (19). Recently, high-throughput phenotypic screens have shown plasmodial ARS to be druggable targets that can be selectively inhibited (16). In this latter work, cladosporin, a fungal secondary metabolite, was found to target the cytosolic lysyl-tRNA synthetase (LysRS) of the malaria parasite. Antimalarial ARS-directed drug design has also been applied satisfactorily against apicoplastic and cytosolic isoleucyl-tRNA synthetase (IleRS) (17) and apicoplastic LysRS (18). However, all previously identified antimalarial drugs targeting ARS either lacked potency (18) did not show in vivo antimalarial activity (17) or showed poor oral bioavailability (16).

Significance

Malaria remains one of the main health threats in the developing world, with staggering social and economic costs. Resistance to artemisins, the main pharmacological tool currently available against malaria, has been widely reported. Borrelidin, a natural compound that inhibits threonyl-tRNA synthetase, has long been studied for its antibacterial and antiparasitic properties, but undesirable toxic effects prevented its further clinical development. Here we present a group of borrelidin derivatives that retain their ability to inhibit *Plasmodium* threonyl-tRNA synthetase but not its human homolog. Furthermore, we demonstrate, for the first time to our knowledge, that these compounds are capable of effectively clearing a *Plasmodium* infection in animals, curing malaria with a potency equivalent to reference drugs such as chloroquine.

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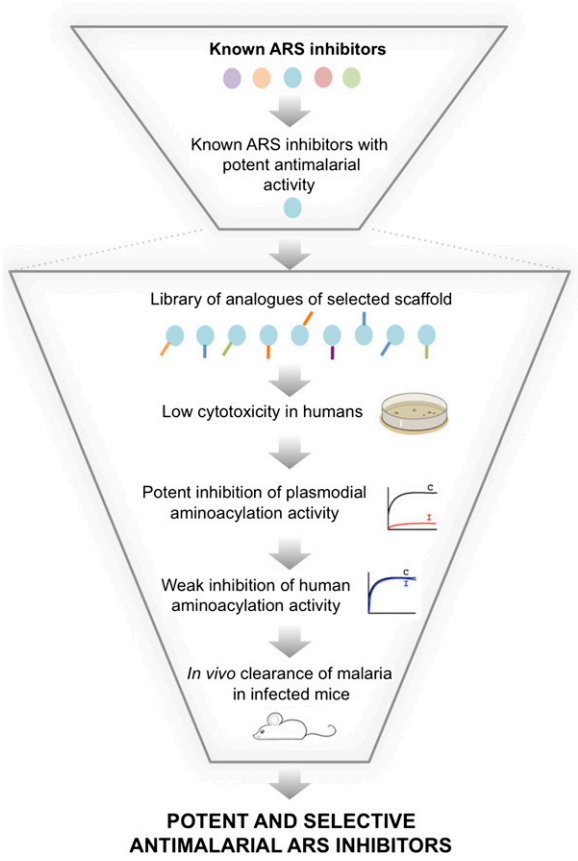


Fig. 1. Antimalarial drug design strategy and pipeline used for identifying potent and selective ARS inhibitors.

To further explore plasmodial ARS as antimalarial drug targets we tested a battery of known ARS inhibitors against *P. falciparum* cell cultures. Among these, we found that borrelidin exhibits excellent antimalarial activity, as previously reported (20–22). Borrelidin is a noncompetitive inhibitor of both bacterial and eukaryotic threonyl-tRNA synthetases (ThrRS) (23) and exhibits antiangiogenic (24–26), antimalarial (21, 22), and antimicrobial (27) properties. The antimalarial activity of borrelidin is thought to arise from inhibition of ThrRS, which in *Plasmodium*, is present as a single enzyme dually targeted to cytosol and apicoplast (28). However, its high cytotoxicity in humans limits its use (29). Moreover, a borrelidin analog was recently described with an antimalarial activity 10-fold lower than borrelidin, but with a cytotoxic effect against human cells 70-fold lower than the parent compound (30). These reports highlight the interest in borrelidin analogs and confirm the potential that analogs may hold for the discovery of compounds with in vivo activity.

We now tested a series of borrelidin analogs for antimalarial activity both in vitro and in vivo and for its cytotoxicity in human cells (Fig. 1). Our results show that several analogs possess higher selectivity than borrelidin and that specific modifications of the borrelidin scaffold can lead to increased selectivity without losing antimalarial activity. Importantly, two of these compounds, BC196 and BC220, efficiently clear the malarial infection in *P. yoelii*-infected mice, leading to 100% mice survival.

Results

Screening of Known ARS Inhibitors on *P. falciparum*. We first screened a battery of known ARS inhibitors against in vitro cultures of *P. falciparum* 3D7 (Table 1). The collection of inhibitors included (*i*) analogs of the native ligands or reaction intermediates (mechanism-based inhibitors); (*ii*) natural product inhibitors and their derivatives; and (*iii*) novel scaffolds targeting ARS. For each compound, IC_{50} values were computed both at 48 and 96 h to distinguish between apicoplastic and cytosolic ARS inhibition. It has been described that the inhibition of apicoplast maintenance or replication causes a phenotype known as "delayed death" (31), in which the parasites do not show growth inhibition during the first asexual cycle of drug treatment (48 h) but die in the second asexual cycle (96 h) even if the drug is removed after the first cycle (32). Therefore, we used this phenotype to distinguish between apicoplastic and cytosolic ARS inhibition.

Our results show that most analogs of the native ligands or reaction intermediate were active against plasmodial ARS in the nanomolar range (Table 1). Their similar IC_{50} values at both 48 and 96 h suggest that these compounds inhibit cytosolic ARS. Natural product ARS inhibitors were also screened for antimalarial activity (Table 1). Among these, mupirocin was relatively inactive at 48 h [IC_{50} (48 h) = 257 μM], but active in the nanomolar range during the second asexual cycle [IC_{50} (96 h) = 93 nM]. This observation is in agreement with previous results (17) and consistent with its high selectivity toward bacterial-type enzymes (33, 34), such as the apicoplast-targeted isoleucyl-tRNA synthetase (IleRS-2). This phenomenon was observed even when mupirocin was removed from the culture after the first cycle of incubation. Cispentacin, a proline analog that inhibits prolyl-tRNA synthetase, was found to be a weak inhibitor of *Plasmodium* cultures even though it was previously shown to effectively protect against systemic *Candida albicans* and *Cryptococcus neoformans* infections (35). This discrepancy could be due to the fact that, in fungi, cispentacin accumulates at high intracellular levels through an active transport mechanism (36) that might be missing in *Plasmodium*. The results for another natural product, borrelidin, are described below.

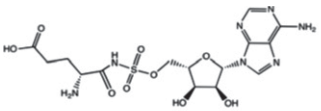
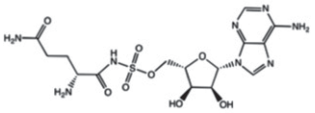
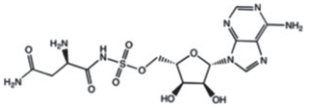
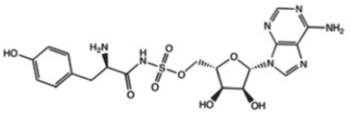
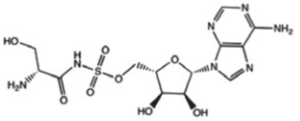
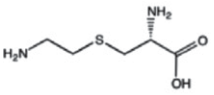
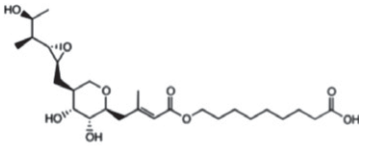
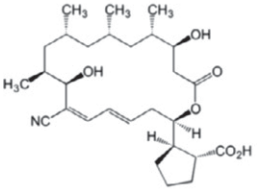
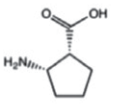
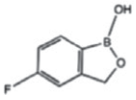
We also show that benzoxaborols have antimalarial activity, in agreement with recently published results (37). Benzoxaborols, such as the well-described AN2690 (38, 39), specifically inhibit the leucyl-tRNA synthetase (LeuRS) editing site by forming a covalent bond with the terminal adenosine (A76) of the $tRNA^{Leu}$, thus trapping the $tRNA^{Leu}$ in the editing active site and causing the death of the pathogen. AN2729 (Anacor Pharmaceuticals) is another member of these novel boron-containing drugs that presumably inhibits the editing domain of LeuRS (38, 39). We investigated the effect of AN2729 on parasite growth and showed that it is capable of inhibiting *P. falciparum* cultures at low micromolar concentrations at 48 h (Table 1), indicating that boron-based compounds can potentially be used as antimalarial drugs.

Borrelidin as an Antimalarial Scaffold. The most potent antimalarial activity was shown by borrelidin, a potent macrolide known to inhibit mammalian, bacterial, and protozoan threonyl-tRNA synthetases (ThrRS) (21, 22, 27, 30, 40). With an IC_{50} of 0.97 nM, borrelidin is a more potent antimalarial compound than artemether, artesunate, or chloroquine (21, 22). Its activity is thought to arise from inhibition of ThrRS (41). Previous reports have shown that borrelidin is a noncompetitive inhibitor of ThrRS with respect to threonine and inhibits the amino acid activation step (23).

Threonyl-tRNA Synthetase Is the Target of Borrelidin in *P. falciparum*. Genetic selection of *Escherichia coli* borrelidin-resistant mutants showed that borrelidin binds to a hydrophobic region proximal to the zinc at the active site of ThrRS (23). Indeed, the interaction between the two molecules relies on a complex network of hydrophobic and polar interactions. The fact that other ARSs do not have a similar cavity in their active site may explain why borrelidin only inhibits ThrRS and not any other ARS.

To determine whether the decrease in parasitemia caused by borrelidin is due to the inhibition of plasmodial ThrRS, we first

Table 1. Inhibitory activity of known ARS inhibitors tested on *P. falciparum* cultures

Structure	Inhibitor	Target	Gene ID	IC_{50} (48 h)	IC_{50} (96 h)	Fold 96/48 h
Native ligand analogs						
	Glu-SA	Glutamyl-tRNA synthetase	PF13_0257/ MAL13P1.281	372.2 nM	463.5 nM	1
	Gln-SA	Glutaminyl-tRNA synthetase	PF13_0170	172.4 nM	150.3 nM	1
	Asn-SA	Asparaginyl-tRNA synthetase	PFB0525w/PFE0475w	88.3 nM	73.4 nM	1
	Tyr- SA	Tyrosyl-tRNA synthetase	MAL8P1.125/ PF11_0181	98.5 nM	84.6 nM	1
	Ser-SA	Seryl-tRNA synthetase	PF07_0073/PFL0770w	39.5 nM	16.9 nM	2
	Thialysine	Lysyl-tRNA synthetase	PF13_0262/PF14_0166	484.8 μM	154.3 μM	3
Natural product inhibitors						
	Mupirocin	Isoleucyl-tRNA synthetase	PF13_0179/PFL1210w	257.0 μM	93.0 nM	2,763
	Borrelidin	Threonine-tRNA synthetase	PF11_0270	1.24 nM	0.97 nM	1
	Cispentacin	Prolyl-tRNA synthetase	PFL0670c/PFI1240c	573.4 μM	462.8 μM	1
Novel scaffolds						
	AN2729*	Leucyl-tRNA synthetase	PFF1095w/PF08_0011	1.03 μM	0.68 μM	2

*Structure of benzoxaborols corresponds to AN2690.

performed multiple sequence alignments of ThrRS sequences and verified that the cluster of amino acids binding borrelidin in *E. coli* ThrRS is also conserved in *P. falciparum* ThrRS (Fig. S1*A*). We then confirmed that the aminoacylation activity of *P. falciparum* ThrRS is indeed inhibited by borrelidin using in vitro aminoacylation assays with and without borrelidin (Fig. S1*B*).

Table 2. In vitro inhibitory activities of borrelidin analogs both in *P. falciparum* cultures and human HEK293 cells

Compound	IC_{50} 48 h (nM)	IC_{50} 96 h (nM)	IC_{50} 72 h (μM except where noted)	IC_{50} 72 h (Hs)/IC_{50} 96 h (Pf)
BC194	3.88	3.49	13.32	3,816
BC195	7.15	4.48	72.06	16,084
BC196	6.73	4.40	59.75	13,579
BC197	93.68	76.71	> 100	>1,303
BC218	17.40	25.00	4.61	184
BC219	25.35	18.59	53.63	2,884
BC220	44.36	23.71	95.99	4,048
BC221	36.08	24.19	16.87	697
BC236	57.30	55.21	24.02	435
BC239	8.13	8.05	16.76	2,081
BC240	9.63	17.25	92.26	5,348
BC249	11.06	25.01	17.14	685
BC253	100.60	56.95	> 100	>1,755
Borrelidin	1.24	0.97	345	355

Also see Table S1.

Borrelidin Analogs Show Higher Selectivity and Lower Cytotoxicity. Previous results, in addition to those presented in this study, point to borrelidin as a potential antimalarial agent (IC_{50} = 0.97 nM). However, borrelidin also causes human cell toxicity at low concentrations (IC_{50} = 345 nM), presumably due to inhibition of human ThrRS (Table 2). Therefore, we decided to screen a library of borrelidin analogs (Fig. 2) to search for more potent and selective antimalarial compounds. These analogs include compounds generated by biosynthetic engineering (24), semisynthesis (42) and a combination of these methods.

We found that 13 of the analogs tested are active in vitro, although with reduced potency compared with borrelidin (Table 2 and Table S1). For some of the compounds, the decrease in potency was only moderate (less than fivefold). Importantly, most of these analogs also appeared to be less cytotoxic than borrelidin (Fig. 3). For instance, BC195 displays a 16,000-fold difference in inhibitory activity when tested on *P. falciparum* compared with human cells, whereas the difference for borrelidin was only 355-fold. Overall, these results suggest that some borrelidin analogs are up to 50 times more selective than borrelidin (Fig. 3 and Table 2).

Borrelidin Analogs Clear Malaria in *P. yoelii*-Infected Mice. Among the 13 borrelidin derivatives that presented antimalarial activity at 100 nM, we selected the five most selective compounds (based on IC_{50} values) for in vivo studies with *P. yoelii*-infected mice (BC194, BC195, BC196, BC220, and BC240). Each compound was tested at two different concentrations: 0.25 (the effective dose of borrelidin) and 6 mg/kg per day (the effective dose of chloroquine) (Table 3). For each compound and dose, the average parasitemia was measured, and the survival time was monitored for up to 20 d after drug treatment (Fig. 4*B*).

Our results show that two of the five compounds tested, BC196 and BC220, yield 100% survival at 6 mg/kg per day (Table 3). The most promising compound, BC220, completely clears the parasitemia at 6 mg/kg per day using the 4-d suppresive test (*Materials and Methods*), which is comparable to parasite clearance by chloroquine. Indeed, this compound already induces 80% mice survival and a very good suppression of parasitemia at 0.25 mg/kg

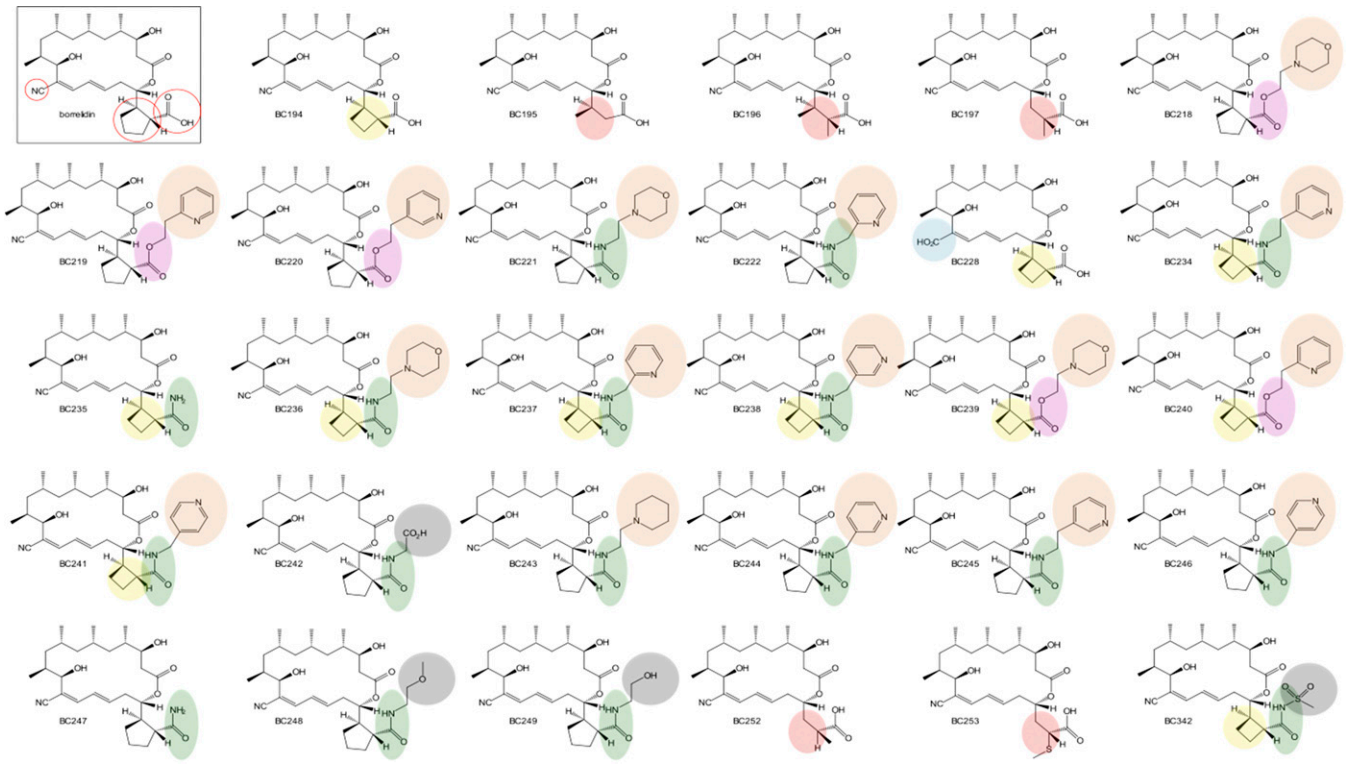


Fig. 2. Library of borrelidin analogs tested on cell-based assays of *P. falciparum* cultures. The structure of borrelidin is shown with the three substituents modified in the analog library circled in red. The structural variation of the analogs is color coded red according to the substitutions explored: (*i*) the nitrile group was substituted for a carboxylic acid moiety (cyan); (*ii*) the cyclopentane moiety was substituted for a cyclobutane (yellow) or aliphatic side chain (red); and (*iii*) the carboxylic acid group was esterified (pink) or amidated (green). Further, the new ester and amide moieties included a range of smaller substituent side chains including carboxylate isosteres (gray) or heterocycles (orange), both aromatic and nonaromatic in nature.

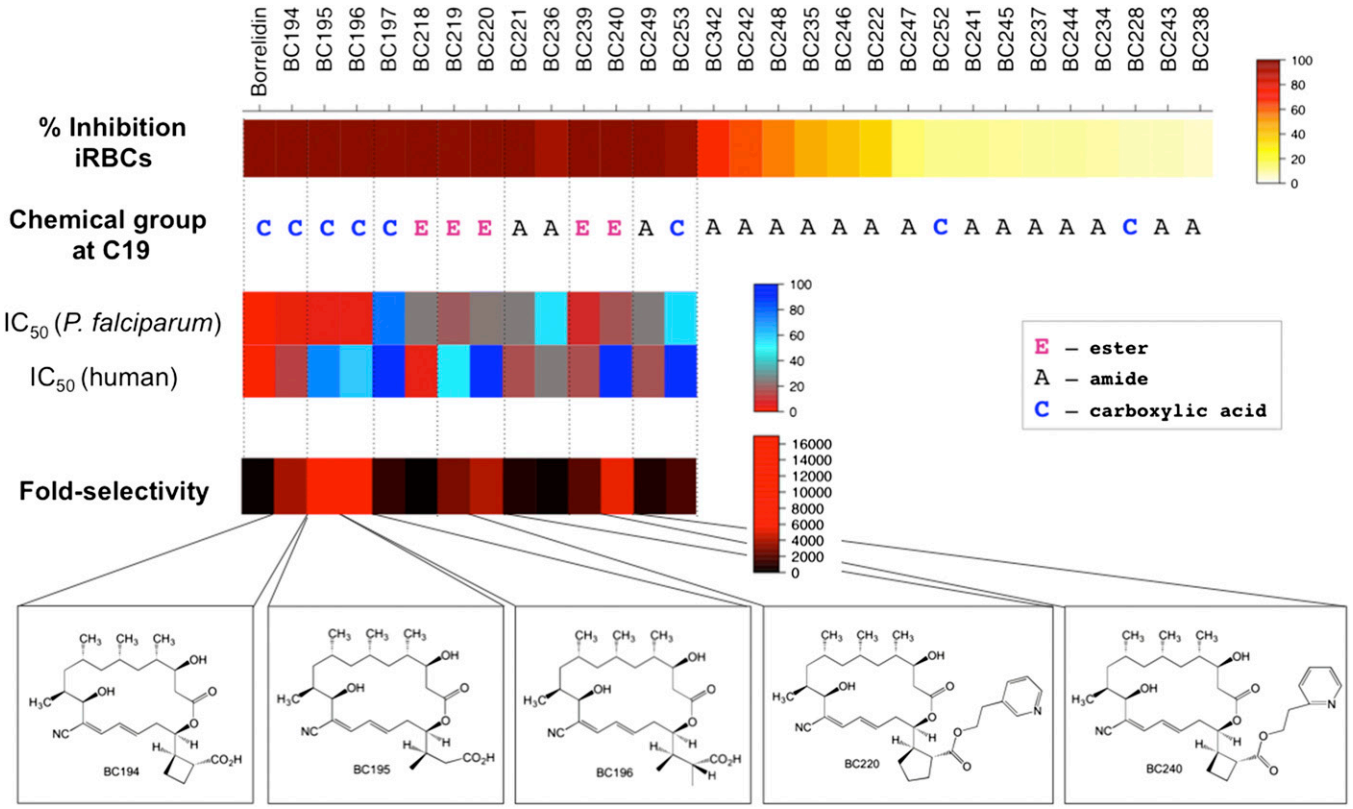


Fig. 3. Antimalarial activity of the library of borrelidin analogs. In vitro antimalarial activities of borrelidin derivatives tested at 100 nM. Borrelidin was included as positive control. Compounds inhibiting over 80% at 100 nM (13 among the 30 compounds tested) were considered to be active and were selected for IC_{50} determination in both *P. falciparum* iRBC (nanomolar) and HEK293T cultures (micromolar). The fold selectivity comparison of the 13 selected borrelidin analogs compared with borrelidin is shown. See also Table S1.

per day. Interestingly, this was not the most potent analog tested in in vitro assays, suggesting that compound bioavailability or other ADME (absorption, distribution, metabolism and excretion) properties affect the activity of borrelidin analogs in vivo.

Finally, it should be noted that all infected mice treated with the borrelidin derivatives that cleared parasitemia developed immunity that protected them from reinfection on further parasite challenge 75 d after the primo-infection. At reinfection, microscopic parasitemia or any perceptible clinical sign was not detected.

In Vitro Aminoacylation Assays Confirm That BC196 and BC220 Are More Selective Compared with Borrelidin. To confirm whether the decrease in cytotoxicity was due to a decreased inhibition of borrelidin analogs on the human enzyme, we directly measured the

Table 3. In vivo i.p. antimalarial activities and curative activity of the five most selective borrelidin derivatives (BC194, BC195, BC196, BC220, and BC240) against *P. yoelii yoelii* 17XL-infected mice

Compound	Dose (mg/kg/d)	No. of mice	Average percentage parasitemia*	Average percentage suppression	Percentage survival	Survival days in fatal cases
Negative control	—	5 (×2)†	87.0 ± 2.6	0	0	4.8 ± 0.3
Chloroquine	6	5	2.2 ± 2.4	97.5 ± 2.8	100	—
Borrelidin	0.25	5 (×2)†	1.3 ± 0.3	98.9 ± 0.5	100	—
BC194	0.25	5	71.5 ± 4.3	17.8 ± 4.9	0	5.0 ± 0.1
	6	5	9.0 ± 2.0	89.7 ± 2.2	40	8.3 ± 0.3
BC195	0.25	4‡	87.2 ± 3.0	1.7 ± 2.1	0	4.7 ± 0.7
	6	5	41.7 ± 13.3	52.0 ± 15.3	25	6.3 ± 0.3
BC196	0.25	5	75.8 ± 2.6	12.8 ± 3.0	0	5.2 ± 0.2
	6	5	4.7 ± 2.0	94.6 ± 2.3	100	—
BC220	0.25	5	3.3 ± 1.2	96.2 ± 1.3	80	9.0 ± 0.1
	6	5	0.1 ± 0.1	99.9 ± 0.2	100	—
BC240	6	5	19.1 ± 5.5	78.1 ± 6.3	60	7.5 ± 0.3

*Parasitemia was determined by microscopic examination of Wright's stained blood films taken on day 4 p.i. Results are presented as the mean value ± SEM.
†A preliminary experiment was performed with borrelidin-treated and negative control mice to calculate the suppression activity and the group size required (*Materials and Methods*). Results from the two groups of five are displayed.
‡One mouse from this group was lost during manipulation, and, due to the low suppression activity and the homogeneous results, the experiment was not repeated.

ability of the three top-ranked borrelidin analogs (BC194, BC196, and BC220), based on the in vivo results, to inhibit the aminoacylation reaction of both *P. falciparum* and human ThrRS.

P. falciparum encodes a single ThrRS that acts both in the cytosol and the apicoplast (28). In contrast, human cells encode two distinct ThrRSs that act separately in the cytosol and the mitochondria. (Fig. S2*A*). The degree of conservation of the active sites of the two human enzymes suggests that both can be sensitive to borrelidin (Fig. S2 *B* and *C*). Nevertheless, the fact that the human cytosolic ThrRS is expressed at levels 300-fold higher compared with the mitochondrial ThrRS suggests that the major target that is being evaluated in cell-based assays in human cells is the cytosolic ThrRS (Fig. S2*A*). Therefore, aminoacylation assays were performed using purified human cytosolic ThrRS as enzyme source. As expected, our results confirm that both BC196 and BC220 are more selective than borrelidin and that the observed decrease in cytotoxicity is likely due to a decreased inhibition of human ThrRS (Fig. 4*C* and Table 4).

Importantly, we also find that there is a clear structure-activity relationship for the borrelidin analogs (Figs. 2 and 3). The most selective compounds were found to have either changes in the native cyclopentane ring that conferred different stereochemical and rotational constraints on the carboxylic acid group (e.g., switch to cyclobutyl, BC194/5/6 series) or had an ester bond substituent of the carboxylic group (BC220/240). Indeed, all analogs containing ester bond substitutions (BC219/220/239/240) not only retained strong antimalarial activity but also showed a significant decrease in cytotoxicity (more than eightfold) compared with borrelidin (Fig. 3 and Table 2). In contrast, amide derivatives showed little or no antimalarial activity at the tested concentrations. Remarkably, this was also true even in those cases where the unique difference between two given analogs was exclusively the amide bond in comparison with an ester bond linkage (e.g., BC220 compared with BC245).

In Silico Analysis Does Not Explain the Selectivity of Borrelidin Analogs. Previous work identified borrelidin as a noncompetitive inhibitor with respect to threonine and ATP in *E. coli* ThrRS (23). Thus, borrelidin was predicted to bind close to the active site, but without overlapping the binding site of either ATP or threonine. Recently, the X-ray structures of borrelidin-bound *E. coli* and human ThrRS have been obtained and they reveal that borrelidin binds to the active site of the enzyme. The structural explanation for the noncompetitive nature of the inhibitor remains, however, undetermined.

To understand the structural reasons behind the selectivity of certain borrelidin analogs, we used in silico docking to predict their binding modes on both *P. falciparum* and human ThrRS. We first built a homology model of *P. falciparum* ThrRS using the borrelidin-bound ThrRS structures as templates. We then in silico docked BC220 and BC196, the two borrelidin derivatives that showed best in vivo activity and selectivity. As expected, binding of the borrelidin moiety of the two analogs overlapped with the binding mode of borrelidin itself (Fig. 4*E*). However, the structural differences found in these two compounds cannot explain the differences in selectivity observed compared with borrelidin. The high conservation of *P. falciparum* and human ThrRS at their borrelidin binding sites led to very small differences in terms of docking scores (a proxy for binding affinity) compared with borrelidin (Fig. 4*D*), and therefore our in silico

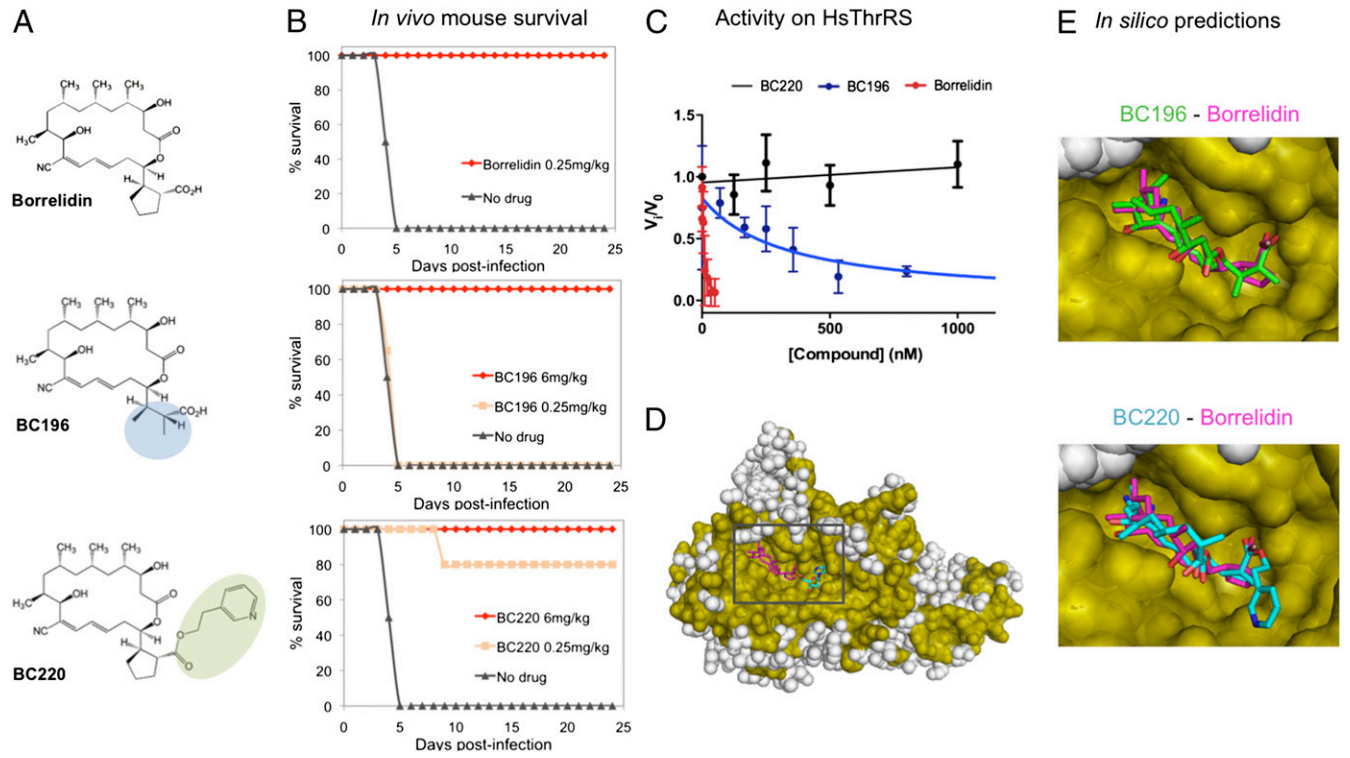


Fig. 4. In vitro and in vivo activities of BC196 and BC220. (*A*) Chemical structure of BC196 and BC220 compared with borrelidin. (*B*) In vivo mice survival of *P. yoelii*-infected mice treated with the selected subset of borrelidin analogs. Percentage of mice survival of *P. yoelii*-infected mice, measured over 20 d after drug treatment. Chloroquine (Cq) has been used as positive control (Table 3). (*C*) Comparison of the inhibitory activity of the aminoacylation reaction catalyzed by human cytosolic ThrRS. (*D*) Homology model of *P. falciparum* ThrRS. Residues have been colored by its conservation with the human cytosolic ThrRS: conserved -same residue- (yellow), not conserved -different residue- (white). Borrelidin is shown in magenta, whereas AMP is shown in cyan. (*E*) Predicted binding mode of BC196 (green) and BC220 (cyan), superimposed with the predicted binding mode of borrelidin (magenta) in *Homo sapiens*. Predicted binding modes are identical to those found in *P. falciparum* ThrRS (not shown in figure).

analysis does not provide an explanation for the selectivity that we observe. An explanation for this discrepancy is that the differences in selectivity are not due to differences in ligand binding affinity but are instead due to different ADME properties of the analogs, such as drug permeability, which may make these molecules more selective despite maintaining a similar binding mode for ThrRS.

BC220 Is Not Cleaved to Borrelidin by Esterases. The observation that all borrelidin analogs with ester substitutions show strong antimalarial activity both in vivo and in vitro, but exhibit higher K_i values against ThrRS (e.g., BC220) than other analogs, suggests that they may actually be acting as prodrugs. Importantly, cleavage of the ester linkage of borrelidin derivatives would yield borrelidin as a result, which is more potent than any of its analogs at inhibiting PfThrRS.

To explore this possibility, we carried out time course experiments with infected red blood cells (RBCs) to determine whether BC220 was converted to borrelidin in the cultures. We incubated human RBCs and human RBCs infected with *P. falciparum* with borrelidin and BC220. Duplicate samples were recovered at 30 min, 3 h, and 27 h, following the protocol described (*Materials and Methods*) for the extraction of metabolites from erythrocytes. All of the resulting samples were analyzed by HPLC with photodiode array detector (HPLC-PDA) to determine the presence or absence of borrelidin and BC220. We find that borrelidin remains stable in the cultures and is not degraded in the course of the experiment (Fig. 5 and Table S2). Similarly, BC220 remains stable in all of the samples analyzed (Table S2), and it is not converted to borrelidin at least within the detection limits of our equipment. These results suggest that the biological effect of BC220 upon *P. falciparum*-infected RBCs in culture is not due to its conversion to borrelidin by RBC or plasmodial esterases (Fig. S3).

Discussion

Resistance of malaria parasites to available drugs continues to grow, limiting our ability to control this serious disease. Although our understanding of the parasite's biology has increased now that the sequence of the *Plasmodium* genome is available (43), few new drug targets or classes of drugs have been validated (44, 45), and current therapies seem to be insufficient for malaria elimination (45–47). Major antimalarial efforts during the last few years include strategies as diverse as the use of combination therapy; the development of analogs of existing agents; the discovery of natural products inhibitors; compound reprofiling; the evaluation of drug resistance reversers; and the consideration of new chemotherapeutic agents (30, 31, 44, 48, 49).

Among the latter, several high-throughput in vitro screening campaigns against *P. falciparum*-infected RBCs (iRBCs) have been published. A library of two million compounds from GlaxoSmithKline's chemical library was screened against *P. falciparum* cultures (50). Of these, 13,500 inhibited parasite growth and more than 8,000 also showed potent activity against a multidrug resistant strain. The public availability of this large set of potent and drug-like antiplasmodial structures provides reasonable staring points for further drug development. In a similar fashion, chemical genetic approaches to assay more than 300,000 chemicals (51) and more than 12,000 natural products (52) against *P. falciparum* iRBCs have been performed. Although these approaches are extremely powerful to identify novel potent antimalarial scaffolds, the lack of knowledge of their respective molecular targets hinders hit-to-lead optimization during the drug development process.

Table 4. In vitro inhibition of the aminoacylation reaction catalyzed by *P. falciparum* and human ThrRS

Compound	Pf ThrRS [K_i (nM)]	Hs ThrRS [K_i (nM)]	Selectivity compared with borrelidin (fold)
BC-194	120	4.09*	0.87
BC-196	440	208.20	12.17
BC-220	4,750	No inhibition†	Not applicable
Borrelidin	140	5.44	1.00

*Data from Williams et al. (26).
†No inhibition found at the maximal concentration tested (10 μM).

Aminoacyl-tRNA synthetases are essential and druggable targets whose activity can be easily measured through in vitro aminoacylation assays (13, 15). Although underexploited for many years, recent work has shown that plasmodial ARS are not only druggable enzymes (17, 18) but also that selective inhibition of these enzymes versus their human homologs is feasible (16). In this work, we evaluated and tested a series of known ARS inhibitors on *P. falciparum* cell cultures and explored their ability to inhibit parasite replication in vitro. Among these, borrelidin is the most potent antimalarial inhibitor (IC_{50} = 0.97 nM). However, borrelidin is not selective enough for clinical applications (21). For this reason we decided to test borrelidin analogs to identify more selective inhibitors.

Recently, a series of borrelidin derivatives was explored for their ability to inhibit cell cultures of *P. falciparum* iRBCs (30). Borrelidin analogs were produced semisynthetically by (*i*) diacetylation at C3 and C11; (*ii*) several types of modifications at C22 (on the cyclopentane ring); and (*iii*) reduction of the diene functionality at C12–C15. The study found that analogs with modifications at the carboxyl group of C22 showed lower in vitro cytotoxicity, which is in accordance with our results. However, they did not test the inhibitory activity against the aminoacylation reactions catalyzed by PfThrRS and HsThrRS or ascertain whether the synthesized compounds possessed in vivo activity. Here we show not only that some borrelidin analogs have greater than 10-fold selectivity over borrelidin, but also that they can retain strong in vivo activity. Indeed, BC220 shows strong in vivo activity with 100% mice survival, a parasite clearance comparable to chloroquine and improved immunological profile on reinfection (20).

Despite its increased K_i, BC220 clears malaria from *P. yoelii*-infected mice with 100% mice survival. Some drugs, once administered to cell cultures or whole organisms, are converted to their active form through normal metabolic processes. For example, salicin is cleaved by esterases to release salicylic acid, the active molecule of aspirin (53). In this line of thought, we first hypothesized that ester borrelidin derivatives, such as BC220, may actually be cleaved by esterases, releasing borrelidin. However, we have shown that BC220 is stable in RBC cultures and that it is not converted into borrelidin. This observation indicates that BC220 is a promising lead antimalarial compound that combines activity, safety, and chemical stability in vitro. Another potential explanation for our results with ester borrelidin derivatives is that these compounds may offer increased selectivity due to better permeability in infected erythrocytes. Malaria parasites have been shown to increase erythrocyte permeability to ions, organic solutes, and antimalarial drugs through new permeation pathways that are associated to *P. falciparum* infection (54–57). Further work is needed to determine the role of permeability differences in the selectivity of borrelidin analogs.

Although it is a formal possibility, existing data indicate that nonspecific adsorption of borrelidin to supernatant protein is not a factor in our data. The K_i of borrelidin for human ThrRS is 5 nM, whereas the K_i for secondary targets such as splicing factor FBP21 or CDKs, are more than 1000-fold higher, approximately of 10 μM (26, 58). Moreover, borrelidin exhibits comparable potencies in the HUVEC (human umbilical vein endothelial cells) tube formation and chicken chorionic allantoic membrane

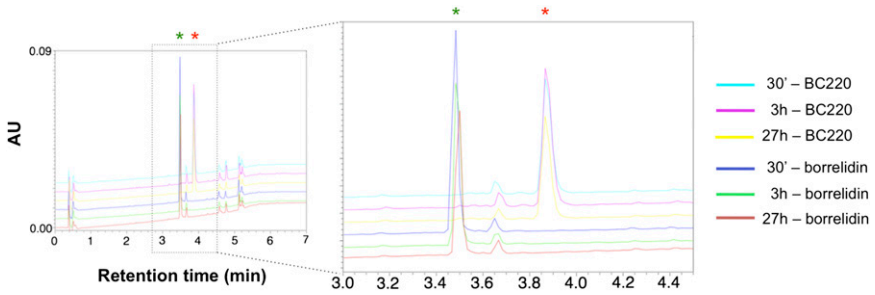


Fig. 5. HPLC-PDA analysis of borrelidin and BC220 stability in *P. falciparum*-infected RBC cultures. HPLC-PDA traces of extracted metabolites from *P. falciparum*-infected RBC treated with BC220 (cyan, magenta, yellow) and borrelidin (blue, green, brown), respectively. The traces do not vary as incubation time increases. The peak corresponding to BC220 is highlighted with a green asterisk, and the peak corresponding to borrelidin is highlighted with a red asterisk. See also Table S2 and Fig. S3.

models, the concentration and composition of extracellular proteins of which is vastly different (26). It has also been shown that a point mutant in the active site of human ThrRS fully blocks the antiangiogenesis effects of the borrelidin analog BC194, whereas the corresponding substitution in *E. coli* ThrRS similarly confers a 4-log increase in resistance to borrelidin (23, 26). Thus, previously published data demonstrate that borrelidin and some of its analogs display comparable inhibitory properties against ThrRS despite being tested under very different assay conditions. All of these argue against off target effects being the basis of the selectivity differences reported here.

Our results show that borrelidin can be used as a scaffold for antimalarial drug design and validate threonyl-tRNA synthetase as a druggable antimalarial drug target. We further show that two borrelidin analogs, BC196 and BC220, have antimalarial activity both in vitro and in vivo. Importantly, their increased selectivity toward *P. falciparum* iRBCs is comparable to that found in commercially available drugs (e.g., mupirocin, 8,000-fold selectivity), and in the case of BC220, its in vivo potency and parasitemia clearance is similar to chloroquine. Moreover, BC220 does not inhibit human cytosolic ThrRS, within the range of concentrations tested, and a 4,000-fold lower concentration of this compound is required to kill *P. falciparum* compared with human cells in in vitro culture assays. We believe that these encouraging results, combined with the recently published structure of borrelidin-bound ThrRS, provide useful guidelines for future structure-based drug design efforts to identify a new generation of borrelidin analogs with further increased selectivity and potency.

Materials and Methods

Reagents. Natural product aminoacyl-tRNA synthetase inhibitors were purchased from the following companies: mupirocin (GlaxoSmithKline), borrelidin (Fluorochem), cispentacin (Acros organics), and thialysine (Sigma). AN2729 was a gift from ANACOR. The library of borrelidin analogs was provided by Biotica Technology and produced using published methods (24, 42). Sulfamoyl adenosine analogs were a kind gift from Magali Frugier (CNRS).

IC_{50} Determinations. IC_{50} determinations were performed with synchronous 3D7-A parasite cultures. Parasites were cultured in B+ human erythrocytes under standard conditions, with RPMI 1640-based medium supplemented with Albumax II and no human serum. To calculate the IC_{50} of the most active compounds, parasitemias were determined by FACS using Syto-11 to discriminate parasitized from nonparasitized RBCs (59). Each sample was diluted 1:100 in PBS, and 0.5 mM Syto-11 in DMSO was added to a final concentration of 0.5 μM. Samples were excited at 488 nm and analyzed using an FC500 flow cytometer. Data analysis was performed with the software package Prism 5.

Cell-Based Drug Inhibition Assays. Initial screens to test the activity of compounds against cultured parasites were performed using the lactate dehydrogenase (LDH) activity assay. To perform the assay 20 μL of sorbitol-synchronized infected erythrocytes (3% hematocrit) in each well of a 96-well plate were mixed with 100 μL of Malstat reagent, 10 μL of 2 mg/mL of nitroblue tetrazolium and 10 μL of 0.2 μg/mL phenylethyl sulfate. After 30min of incubation in the dark the reaction was stopped by adding 100 μL of 5% acetic acid to each well. Absorbance at 590nm was measured on a plate reader to quantify the LDH activity, which is proportional to the parasitemia. Smears were also prepared for each drug assay to visually confirm the results based on absorbance results (microscopy counting of Giemmsa-stained smears). For each compound tested, parasite LDH activity was measured both at 48 and 96 h to determine whether the compound exerts a delayed death. Inhibitory compounds were applied to tightly synchronized cells and analyzed by comparing growth between treated and control cultures. A drug was considered to cause delayed death if it met the two following conditions: (*i*) there was no measurable growth inhibition after 48 h of treatment at drug concentrations 10-fold higher than those needed to inhibit 50% of parasite growth at 96 h; and (*ii*) parasite growth inhibition was insensitive to the presence or absence of drug during the second asexual cycle (31).

Cytotoxicity Assays on Human Cells. Cytoxicity was measured using the Cell Profileration assay WST-1 (Roche) on HEK293T cells. Cells were cultured in a 96-well microplate and incubated for 2–4 h with WST-1. During this incubation period, viable cells convert WST-1 to a soluble formazan salt, which was then quantified at 450 nm with an ELISA plate reader.

Enzyme Preparation. The plasmid (pET28a hctThrRS) encoding N-terminal His_6-tagged human ThrRS was a gift from Dieter Soll (Yale University, New Haven, CT) and used to transform *E. coli* Rossetta 2(DE3) pLysS competent cells (EMD). Cells were cultured in Terrific Broth supplemented with 100 mg/mL kanamycin and 100 mg/mL chloramphenicol at 37 °C and grown to a cell density of $A_{600} = 0.6$. ThrRS expression was induced by overnight incubation with 1 mM isopropyl 1-thio-β-D-galactoside (IPTG) at 15 °C. The bacterial pellet was lysed by sonication in buffer A (20 mM potassium phosphate buffer, pH 8.0, 100 mM KCl, 35 mM imidazole, and 5 mM β-mercaptoethanol) and cleared by centrifugation at 17,000 × *g* for 30 min. Nucleic acids were precipitated by the addition of Protamine sulfate to a final concentration of 0.3%, followed by additional centrifugation. The supernatant was loaded onto a HisTrap FF column (GE Healthcare) equilibrated with buffer A and eluted by an imidazole gradient of 35–250 mM over 20 column volumes. ThrRS containing fractions were identified by SDS/PAGE and GelCode Blue (Thermo Scientific), pooled, and dialyzed into buffer B (100 mM potassium phosphate buffer, pH 6.8, and 5 mM β-mercaptoethanol). The sample was loaded onto a CHT-Tricorn hydroxyapatite column and eluted over 20 column volumes by a gradient of buffer B to buffer C (500 mM potassium phosphate, pH 8.0, and 5 mM β-mercaptoethanol). ThrRS containing fractions were determined by SDS/PAGE, dialyzed into buffer D [20 mM Hepes, pH 8.0, 100 mM KCl, 5 mM β-mercaptoethanol, and 40% (vol/vol) glycerol], and stored at −20 °C.

P. falciparum extracts for aminoacylation assays were prepared by hypotonic lysis of saponin-extracted parasites. In brief, parasite cultures were pelleted by centrifugation and resuspended in two pellet volumes of 0.15% saponin (Sigma) in PBS buffer (for a 0.1% final concentration of saponin). After incubating for 5 min in ice to lyse RBC membranes, intact parasites were recovered by centrifugation and washed twice with PBS. Parasites pellets were resuspended in hypotonic lysis buffer consisting of 20 mM Hepes, pH = 7.8, 10 mM KCl, 1 mM EDTA, and a protease inhibitors mixture (Roche). After incubating for 10 min in ice, extracts were centrifuged, and the supernatant was used for aminoacylation assays.

tRNA^{Thr} Preparation. *E. coli* XL-1 Blue (Stratagene) cells expressing the plasmid pUC18hcttRNA^{Thr}-TGT, coding for human tRNA^{Thr}, were cultured in Luria-Bertani (LB) broth supplemented with 100 mg/mL ampicillin at 37 °C to a cell density of A280 = 0.5. Expression of tRNA^{Thr} was induced with 1 mM IPTG for 14 h. Bacterial pellets were resuspended in 20 mM Tris, pH 7.5, 20 mM $MgCl_2$, and 10 mM β-mercaptoethanol and extracted with water saturated phenol (pH = 4.8). The aqueous fractions were ethanol precipitated, resuspended in 10 mM Hepes and 50% formamide, and resolved using 8 M urea/10% polyacrylamide gel electrophoresis. The tRNA was identified by UV shadowing, electroeluted, and ethanol precipitated. The tRNA pellet was resuspended in 10 mM Hepes and stored at −20 °C. The active concentration of tRNA^{Thr} was determined by plateau charging assay.

Aminoacylation Assays and K_i Determination. The apparent inhibition constant (K_i^{app}) of the various inhibitors for ThrRS was determined using modifications to previously described steady-state aminoacylation procedures (23). Reaction mixtures consisted of 20 mM Hepes, pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 20 μM [^{14}C]-threonine, 180 μM unlabeled threonine, 7.5 μM tRNA^{Thr}, 40 nM ThrRS, and various concentrations of borrelidin, BC196, or BC220. Reactions were incubated for at least 10 min on ice to allow for the slow binding of inhibitors and initiated with the addition of 2 mM ATP. Aliquots were taken at varying time points and spotted onto Whatmann 3MM paper filters presoaked in 5% trichloroacetic acid (TCA). On reaction completion, the filters were washed three times in excess TCA and once in 95% ethanol and dried under a heating lamp. The formation of Thr-tRNA^{Thr} was detected by scintillation counter, and the activity was determined by linear regression of threonyl-tRNA^{Thr} formed per active site per unit time. Values for K_i^{app} were obtained by plotting fractional velocity as a function of inhibitor concentration and fitting the data to Morrison's quadratic equation for tight binding inhibitors (60) using GraphPad Prism software

$$\frac{v_i}{v_0} = 1 - \frac{[E]_T + [I]_T + K_i - \sqrt{\left([E]_T + [I]_T + K_i\right)^2 - 4[E]_T[I]_T}}{2[E]_T},$$

where $[E]_T$ is the concentration of total active enzyme in the reaction, $[I]_T$ is the total inhibitor concentration, and K_i the apparent inhibition constant.

Preclinical Treatments on a *P. yoelii* in Vivo Model of Lethal Malaria. The in vivo study was carried out according to standard protocol following the 4-d suppressive test (61) implemented as previously described (20, 62). Seventy inbred BALB/cAnNHsd pathogen-free female mice, aged 7 wk and weighting 17–20 g (average, 18.34 g; Harlan Laboratories) were housed at random in 50 × 25-cm airy racks containing 10 labeled animals each. Animals were kept under constant standard conditions of light (12:12-h light:dark cycles), temperature (22–24 °C), humidity (~50%), ad libitum diet (2018 Teklad Global 18% Protein Rodent Diet; Harlan Laboratories), and bedding (Lignocel; Rettenmaier & Sohne) at the Animal House of Universidad Complutense de Madrid. The rodent malaria parasite *P. yoelii yoelii* 17XL was kindly provided by Virgilio Do Rosario (Universidade Nova de Lisboa, Lisboa, Portugal) and stored in liquid nitrogen after serial blood passages in mice.

For the present study with borrelidin analogs, to follow the 3Rs principles (63), we used a 99% value of suppression activity as previously obtained with borrelidin against *P. yoelii yoelii* 17XL (20) to calculate the minimal number of animals ($n = 5$) to detect a minimum suppression activity of 25% with 80% of statistical power ($b = 0.2$) and 95% confidence level ($a = 0.05$). BALB/cAnNHsd mice were infected i.p. on day 0 with 2×10^7 *P. yoelii yoelii* 17XL-infected red blood cells between 10:00 and 12:00 AM, using a 30-G 1/2-in. needle at ~15° angle in the lower quadrant of the abdomen off midline.

P. yoelii 17XL-infected mice were selected at random for treatment with borrelidin (0.25 mg/kg), borrelidin analogs (0.25 or 6 mg/kg) and chloroquine (6 mg/kg). The tested drugs were prepared at appropriate doses in a final volume of 100 mL of aqueous vehicle containing 7% Tween-80 and 3% ethanol as previously described (64). Control animals, also selected at random, received aqueous vehicle (100 mL) by the same route. Four-day treatments were carried out at the animal room, starting 2 h after infection. Ad libitum drinking water during the experiment contained 0.05% of p-aminobenzoic acid, which is essential for the parasite growth and therefore for the infection establishment (65). Parasitemia was monitored daily (between 9:00 and 10:00 AM) in each mouse by performing Wright's staining on thin tail blood smears. The number of dead mice was recorded every 12 h from all of the treatment groups to determine the average survival time.

In Silico Analysis of the Selectivity of Borrelidin Analogs. The primary sequence of *P. falciparum* ThrRS was downloaded from the Uniprot database (www.uniprot.org). The 9v12 version of MODELER (66) was used to create the homology model, using as templates borrelidin-bound human and *E. coli* ThrRS structures. The model was manually refined, including corrections of the alignment using the PSI-PRED (67) secondary structure predictions as guideline, followed by a new rebuilding of the model. The final model was analyzed using ProSA (68) and then validated using PROCHECK (69). Both *P. falciparum* ThrRS homology model and human ThrRS were structurally superimposed to the *E. coli* ThrRS structure using STAMP (70). Borrelidin, BC220, and BC196 were docked against *P. falciparum* and human ThrRS using the software Glide v. 5.0 (71). The ligands were prepared with the LigPrep facility of MAESTRO (Schrödinger LLC), whereas the setup of the protein was done with the Protein Preparation Wizard facility. Schrödinger's GlideScore scoring function was used to score and rank the predicted docking poses.

HPLC-PDA Analysis of Borrelidin and BC220 in Erythrocyte Cultures. The stability of borrelidin and BC220 in cultures of RBCs alone or infected with synchonized *P. falciparum* (>6% parasitemia) was tested after 30 min, 3 h, and 27 h of incubation with the drugs. Cultures at the late trophozoite stage were collected after being treated with the drugs for the stated times and prepared for HPLC-PDA analysis using the protocol for liquid-liquid extraction of metabolites from erythrocytes developed by Agilent Technologies (www.agilent.com/chem/metabolomics.)

Borrelidin and BC220 detection was performed using an HPLC-PDA system composed of an Alliance 2695 (Waters) with auto sampler, column oven set at 30 °C, and a photodiode array detector 2996. Empower 2 software was used for instrument control and acquisition data, and an Xbridge C18 column was used for the separation (Waters). The elution system was composed by A:H_2O:HCCOH [99.9:0.1 (vol/vol)] and B: CH3CN [99.9:0.1 (vol/vol)]. Samples were eluted with a linear gradient of 5%B to 100%B in 4.5 min at a flow rate of 1.6 mL/min. Injection volumes were 10 μL and compounds were detected at 257 nm.

Metabolite Extraction from RBC Cultures After Drug Incubation. Synchronized cultures of *P. falciparum* infected and noninfected RBCs were treated with no inhibitor, BC220, or borrelidin. To incubate the parasites the exact amount of time (27 h), 20 μL of 1 mM of water, BC220, or borrelidin was added to 2mL of cell culture at different time points. For each of the three conditions (water/BC220/borrelidin), drugs were added at $t = 0$, 24, and 26.5 h, corresponding to incubation times of $t = 27$ h, 3 h, and 30 min, respectively, both with infected and noninfected RBC cultures. At $t = 27$ h, samples were collected, and metabolites were extracted using the Metabolite Extraction Protocol for Metabolomics Studies of Erythrocytes (Agilent Technologies). Briefly, 0.5 mL of the erythrocyte samples was centrifuged and resuspended in water, incubated in a water bath at 37 °C for 0.5 min, and resuspended in −20 °C methanol; 0.45 mL of chloroform and 0.15 mL of ice cold water were added to each tube. After centrifugation, the top and bottom phases were separated, vacuum dried, and independently analyzed by HPLC-PDA. Duplicates for each sample, condition, and time point were produced and analyzed (Table S2).

Ethics Statement. All animal care and experimental procedures carried out at Complutense University of Madrid complied with Spanish (R.D. 32/2007) and European Union legislation (2010/63/CE), were approved by the Animal Experimentation Committee of this institution, and are reported following the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (62). The number of animals was calculated using Statgraphics Centurion 16.1.18 software (Statpoint Technologies) to provide about 80% of statistical power with 95% confidence level, always following the 3Rs principles.

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