COMMENTARY

The growing pipeline of natural aminoacyl-tRNA synthetase inhibitors for malaria treatment

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ABSTRACT
Malaria remains a major global health problem. Parasite resistance to existing drugs makes development of new antimalarials an urgency. The protein synthesis machinery is an excellent target for the development of new anti-infectives, and aminoacyl-tRNA synthetases (aaRS) have been validated as antimalarial drug targets. However, avoiding the emergence of drug resistance and improving selectivity to target aaRS in apicomplexan parasites, such as Plasmodium falciparum, remain crucial challenges. Here we discuss such issues using examples of known inhibitors of P. falciparum aaRS, namely halofuginone, cladosporin and borrelidin (inhibitors of ProRS, LysRS and ThrRS, respectively). Encouraging recent results provide useful guidelines to facilitate the development of novel drug candidates which are more potent and selective against these essential enzymes.

KEYWORDS
Aminoacyl-tRNA synthetase; borrelidin; cladosporin; halofuginone; malaria; Plasmodium falciparum

Plasmodium parasites are the causative agents of malaria. They have a complex life cycle that includes 2 major phases (liver and blood) in the human host. In 2013, there were about 198 million cases of malaria and an estimated 584 000 deaths. Most deaths are attributable to P. falciparum, one of the 5 Plasmodium species infecting humans. Several malarial drugs are currently on the market to treat uncomplicated or severe malaria. Chloroquine is cheap and widely available, although it is no longer effective in many countries. All other treatments are more expensive. Mefloquine, for example, has a much higher price per treatment and does not eliminate parasites in the liver phase of the disease. Patients with P. vivax malaria are therefore treated with a second drug that is effective against the liver phase, such as primaquine. Doxycycline can be used as prophylaxis against malaria (liver stage of Plasmodium) and, when used in conjunction with a fast acting agent like quinine, is highly effective and used against chloroquine resistant P. falciparum or P. vivax. The artemisinin group of drugs constitutes the best line of treatment currently available. Artemether is effective against the blood phase of both malarial parasites, P. falciparum and P. vivax, and artesunate is widely used for severe malaria.

Treatment of malaria is confronted with significant challenges, such as the lack of an effective vaccine and emergence of drug resistance over time. Widespread drug resistance against commonly used drugs such as chloroquine and pyrimethamine/sulfadoxine (Fansidar) has been reported. In more restricted areas, mefloquine, quinine and halofantrine resistance were also observed. Although there are many effective agents, endoperoxides are the only drug class for which clinically significant resistance has not yet been reported. The first generation endoperoxides includes artemisinin and several semisynthetic derivatives such as arteether, arteether, artesunate and artelicate. Unfortunately, endoperoxides are ineffective in the liver stage, and there are concerns that tolerance to artemisinins may be emerging in Cambodia. There is a real and immediate need to discover new drugs which possess activity against both liver and blood stage malaria parasites.

Many approved drug targets are involved in protein translation. Protein synthesis is a complex, multi-step process involving many enzymes. The majority of antimalarials that block protein synthesis interfere with the ribosome. Tetracyclines, including
doxycycline, prevent the binding of aminoacyl-tRNA by blocking the A (aminoacyl) site of the 30S ribosome. Doxycycline treatment is usually combined with quinine due to the delayed antimalarial effect of doxycycline. This delay is related to its mechanism of action in the apicoplast. The apicoplast is a non-photosynthetic plastid, which possesses a small circular genome and undertakes protein translation (harboring up to 30 self-coded proteins). Aminoacyl-tRNA synthetases (aaRS), which catalyze the attachment of amino acids to their cognate tRNAs, are also good antimalarial drug targets and validated in the clinic.

Mupirocin, an inhibitor of isoleucyl-tRNA synthetase (IleRS), is currently marketed for topical treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infection. Interestingly, mupirocin was also shown to inhibit blood stage of *P. falciparum* infection, probably due to its in vivo instability and its high binding to serum. Thus, IleRS is still a good and validated drug target to be explored.

Halofuginone, an inhibitor of prolyl-tRNA synthetase (ProRS), was approved by the US Food and Drug Administration in 1980 for treatment of apicomplexan parasite infections in chickens and turkeys. Halofuginone has a wide range of indications, such as malaria, cancer, fibrosis-related illnesses and autoimmune diseases. It is a derivate of febrifugine, a long-used antiparasitic agent known to have toxic side effects, and that was shown to be an efficient inhibitor of both erythrocytic and liver stage *P. falciparum*. Relative to febrifugine, halofuginone maintains its efficiency against *Plasmodium* but shows a reduced toxicity against human cells. Nevertheless, halofuginone was found to also inhibit human ProRS. Its mechanism of action on ProRS is ATP-dependent and blocks the formation of the Pro-AMP adenylate complex. ATP orient 2 parts of halofuginone onto dual binding sites of ProRS. One part mimics bound proline and the other mimics the 3' end of the bound tRNA. In this way, halofuginone is able to target 2 binding sites to modulate the activity of ProRS. This raises the possibility that similar inhibitors might be applied to other synthetases.

Recently, cladosporin was identified as an inhibitor of lysyl-tRNA synthetase (LysRS). Cladosporin, also called asperentin, is a natural product and fungal secondary metabolite found in diverse fungi including *Aspergillus flavus* and *Cladosporium cladosporioides*. It is composed of a THP ring (2,6-dissubstituted tetrahydropyran) fused to an isocoumarin moiety. Cladosporin presents a large panel of activities, such as antifungal, insecticidal, plant growth inhibition and antibacterial properties, as well as anti-inflammatory activity in mouse lung tissue. Recently, cladosporin was also shown to be a potent antimalarial molecule acting against intraerythrocytic parasites.

This has been demonstrated against both multidrug-resistant *Plasmodium* strains and liver-stage parasites. Little effect on the growth or viability on human cell lines was observed by cladosporin, with a selectivity index against *P. falciparum* compared to human cells superior to 111 (CC₅₀/IC₅₀ > 111). Cladosporin is an attractive candidate therapeutic agent due to its effect on both liver and blood stages of the parasite, which were described before identification of cladosporin’s target. Hoepfner et al. identified the novel druggable target using reverse genomic approaches and concluded that the KRSI gene is responsible for the cladosporin-induced phenotype. KRSI codes for LysRS enzyme. Biochemical data showed that cladosporin directly inhibits PfLysRS and, moreover, was only weakly active toward its human counterpart. Indeed, cladosporin is > 100-fold more potent against parasite LysRS (IC₅₀ 61 nM) relative to the human enzyme. Despite these attractive characteristics for pre-clinical development, cladosporin is known to have a poor oral bioavailability. Thus additional improvements through further drug development are required.

The recent crystal structure of ternary PfLysRS-lysine-cladosporin complex showed that cladosporin mimics the natural substrate adenosine and thereby occupies the PfLysRS active site. In *silico* docking and competition assays revealed that cladosporin might bind the ATP binding pocket of PfLysRS (Kᵩ of 14 ± 1.4 nM) with a similar binding posture to ATP itself. Additionally, key residues responsible for cladosporin selectivity and specificity were identified and further validated. They are 2 amino acids within the enzyme active site, Val328 and Ser344 (Gln and Thr in human LysRS, respectively). The resolution of the ternary complex provides an interesting tool for drug development via structure-based rational design strategies. Indeed, the observation of the presence of
an ample space within the PfLysRS active site allowed further chemical derivatization of cladosporin, facilitated by the available total synthetic route of the molecule.\textsuperscript{23,24}

Dual inhibitors of both cytosolic and apicoplast targets represent an attractive strategy to reduce emergence of resistance, and combine the slow clearance that results from apicoplast delayed death with the rapidity of action of cytosolic translation inhibition. 37 aaRS have been identified in \textit{P. falciparum},\textsuperscript{25} 23 of which present a signal peptide for import into the apicoplast. Alanyl-, glycyl-, cysteinyl- and threonyl-tRNA synthetases (AlaRS, GlyRS, CysRS and ThrRS) are especially interesting potential targets due to their dual localization.\textsuperscript{26-28}

The natural macrolide antibiotic borrelidin was first isolated from \textit{Streptomyces rocheii} in 1949, and its potent inhibitory activity against bacterial growth was later linked to ThrRS inhibition.\textsuperscript{29} Borrelidin was more recently recovered from a large-scale screen of soil microorganism–derived substances for its ability to block malaria parasite growth \textit{in vitro} and \textit{in vivo}.\textsuperscript{30} Borrelidin displayed an IC50 versus growth of drug-sensitive and resistant strains of \textit{P. falciparum} of 1.8 nM and 1.9 nM respectively, making it a more potent inhibitor than artemether, artesunate or chloroquine. Following subcutaneous administration to infected mice, borrelidin was particularly effective against drug resistant \textit{P. yoelii} strains, and after oral administration displayed an ED50 value 13 times lower than any of the aforementioned established antimalariais.\textsuperscript{30}

Part of borrelidin’s potency against parasite growth may lie in the cellular localization of its target in \textit{Plasmodium}. Genomic and immunohistochemical evidence suggests that ThrRS is simultaneously targeted to both the cytoplasm and the apicoplast.\textsuperscript{26} Jackson \textit{et al.}\textsuperscript{27} showed that ThrRS is expressed throughout parasite blood-stage sexual development, and that borrelidin causes a rapid arrest of parasitic growth. Borrelidin is more active than mupirocin, which specifically targets the apicoplast but not cytoplasmic IleRS isozyme, leading to the ‘delayed death’ phenotype associated with inhibitors of crucial apicoplast enzymes in \textit{plasmodium} species. The authors hypothesized that borrelidin’s antimalarial potency may be attributed, at least in part, to the ubiquitous presence of ThrRS throughout the target cell.

The advantage of dual-targetting synergizes with the strength of the inhibition effect by borrelidin against ThrRS. Borrelidin exhibits tight-binding inhibitor kinetics, with a Ki of approximately 4 nM against ThrRS from several species.\textsuperscript{31} Recent elucidation of the atomic-level structure of borrelidin-bound human or bacterial ThrRS fragments suggests that the inhibitor simultaneously occupies the binding sites of the ATP, tRNA and amino acid substrates.\textsuperscript{32} In addition, borrelidin occupies an induced-fit hydrophobic pocket deep in the active site. Mutagenesis experiments suggest that this fourth borrelidin binding pocket contributes to borrelidin’s inhibitory effect on the aminoacylation reaction.\textsuperscript{32} Although such detailed structural information is not yet available for any \textit{Plasmodium} aaRS, key borrelidin-interacting residues are conserved in the \textit{P. falciparum} enzyme, and borrelidin potently inhibits \textit{P. falciparum} ThrRS aminoacylation activity \textit{in vitro}.\textsuperscript{33}

An additional promising aspect of borrelidin’s activity in rodent malaria infection models is that the compound appears to elicit a particularly strong and long-lasting host immune response against the parasite. Borrelidin proved to be more effective than chloroquine-treatment at protecting mice from \textit{P. yoelii} reinfection 75 d after an initial infectious episode that both drugs had cleared effectively.\textsuperscript{34} Further analysis showed that borrelidin treatment during the first infection leads to an increase in the proportion of trophozoite- vs. ring-stage parasites in the host blood stream, and a corresponding increase in host IgGs targeted at later blood stages.\textsuperscript{34} Borrelidin treatment could therefore have a long term prophylactic effect through potentiation of the host immune response. It would be of particular interest to assess whether this occurs in vulnerable patient groups where the host response is compromised, such as pregnant mothers and children.

Despite its efficacy at inhibiting growth of \textit{P. falciparum}, ameliorating infections in mouse models of malaria, and promoting long-term host immunity, borrelidin’s progress toward the clinic has been stunted due to its toxicity in mammalian cells at relatively low concentrations.\textsuperscript{30} The selectivity index of borrelidin against \textit{P. falciparum} compared to human cells is high (CC50/IC50 > 355).\textsuperscript{33} Recent work showed that borrelidin’s toxicity is related to inhibition of mammalian ThrRS, and that chemical modifications can be made to borrelidin’s structure that improve
selectivity for the *Plasmodium* enzyme and favor inhibition of parasite growth over mammalian cytotoxicity.\(^{33,35}\)

Recently, Novoa *et al.* have described borrelidin derivatives that are more selective and less cytotoxic than the original molecule.\(^{33}\) These compounds have a potent antiparasitic activity both *in vitro* and *in vivo*, and clear malaria from *P. yoelii*-infected mice resulting in 100% mice survival rates. Like with borrelidin, infected mice treated with the derivatives developed immunity which protects them from reinfection with the parasite. These findings offer hope that the structure-based design of borrelidin derivatives may yield new species-specific ThrRS inhibitors with a good safety profile for further preclinical development.

Although several drugs are on the market to treat malaria, the emergence of resistance is now common and poses a growing problem. For the different biological reasons discussed here, aaRSs appear to be promising drug targets against *Plasmodium*. Halofuginone, cladosporin and borrelidin are all active against the erythrocytic and liver stages of the infection, and borrelidin targets both the cytosolic and apicoplast aaRS. The recent findings on borrelidin derivatives, including their ability to induce long-term immunity, open a promising avenue for an innovative therapeutic strategy in our race toward the eradication of malaria.

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No potential conflicts of interest were disclosed.

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