ANNUAL REVIEWS

Annual Review of Microbiology

Targeting Aminoacyl tRNA Synthetases for Antimalarial Drug Development

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Annu. Rev. Microbiol. 2023. 77:111–29

First published as a Review in Advance on April 5, 2023

The *Annual Review of Microbiology* is online at micro.annualreviews.org

https://doi.org/10.1146/annurev-micro-032421-121210

Keywords

aminoacyl tRNA, tRNA charging, antimalarial drug development, reaction hijacking

Abstract

Infections caused by malaria parasites place an enormous burden on the world's poorest communities. Breakthrough drugs with novel mechanisms of action are urgently needed. As an organism that undergoes rapid growth and division, the malaria parasite *Plasmodium falciparum* is highly reliant on protein synthesis, which in turn requires aminoacyl-tRNA synthetases (aaRSs) to charge tRNAs with their corresponding amino acid. Protein translation is required at all stages of the parasite life cycle; thus, aaRS inhibitors have the potential for whole-of-life-cycle antimalarial activity. This review focuses on efforts to identify potent plasmodium-specific aaRS inhibitors using phenotypic screening, target validation, and structure-guided drug design. Recent work reveals that aaRSs are susceptible targets for a class of AMP-mimicking nucleoside sulfamates that target the enzymes via a novel reaction hijacking mechanism. This finding opens up the possibility of generating bespoke inhibitors of different aaRSs, providing new drug leads.

Contents

1. INTRODUCTION

Malaria is a debilitating disease caused by protist parasites of the genus *Plasmodium*. Half the world's population lives in subtropical regions where malaria is endemic. Infection is initiated when a female *Anopheles* mosquito bites a human, injecting infectious sporozoites into the bloodstream. The parasites establish infections in liver cells, where they multiply and are released to invade red blood cells. Some blood stage parasites differentiate into transmissible gametocytes that can be taken up by mosquitoes to complete the life cycle.

The blood stage of the infection is associated with symptoms that range in severity from fever, headache, and nausea to severe anemia, respiratory distress, acidosis, coma, and death (73, 82). In 2021, the most lethal malaria species, *P. falciparum*, infected more than 200 million people, causing enormous illness and loss of productivity and leading to 619,000 deaths (102).

Recent gains in the fight against malaria have stalled or been reversed due to disruptions to services during the COVID-19 pandemic, with 16 million more malaria cases and 51,000 more deaths in 2021 compared to 2019 (102). These problems are exacerbated by loss of efficacy of current antimalarial treatments, with ~50% treatment failure of current artemisinin-based combination therapies in some regions in Southeast Asia (2, 52, 90, 97). Recently, resistance to artemisinins has been clinically validated in Africa (7), where most malaria deaths occur.

The peak body for antimalarial drug development, Medicines for Malaria Venture (MMV), has called for the development of new antimalarial compounds that meet their published target product profiles (14). MMV recommends that new compounds have a novel mechanism of action, be active against all currently known drug-resistant parasite strains, and have a low propensity for resistance development. The drug candidates should reduce the blood stage parasite burden

quickly, in order to save lives, but also remain in the circulation long enough to enable parasite clearance. They should preferably have liver stage activity so that they can be used for prophylaxis, and activity against sexual blood stages to prevent parasite transmission. The candidates should preferably also kill *Plasmodium vivax*. They should have a low cost of goods and be orally bioavailable. Ideally, they would be effective in a single oral dose, providing single-exposure radical cure and prophylaxis (SERCaP) for the treatment of uncomplicated malaria in adults and children. It is recognized that these are daunting criteria (14, 74).

2. AMINOACYL tRNA SYNTHETASES—CRITICAL HOUSEKEEPING ENZYMES

When a ribosome matches a particular mRNA codon with a tRNA anticodon, it relies on the tRNA being loaded with the correct amino acid. The fidelity of protein synthesis is determined upstream by a series of aminoacyl-tRNA synthetases (aaRSs). These enzymes catalyze the charging of tRNAs with their cognate amino acid, in a two-step reaction (**Figure 1*a***). In the first step,

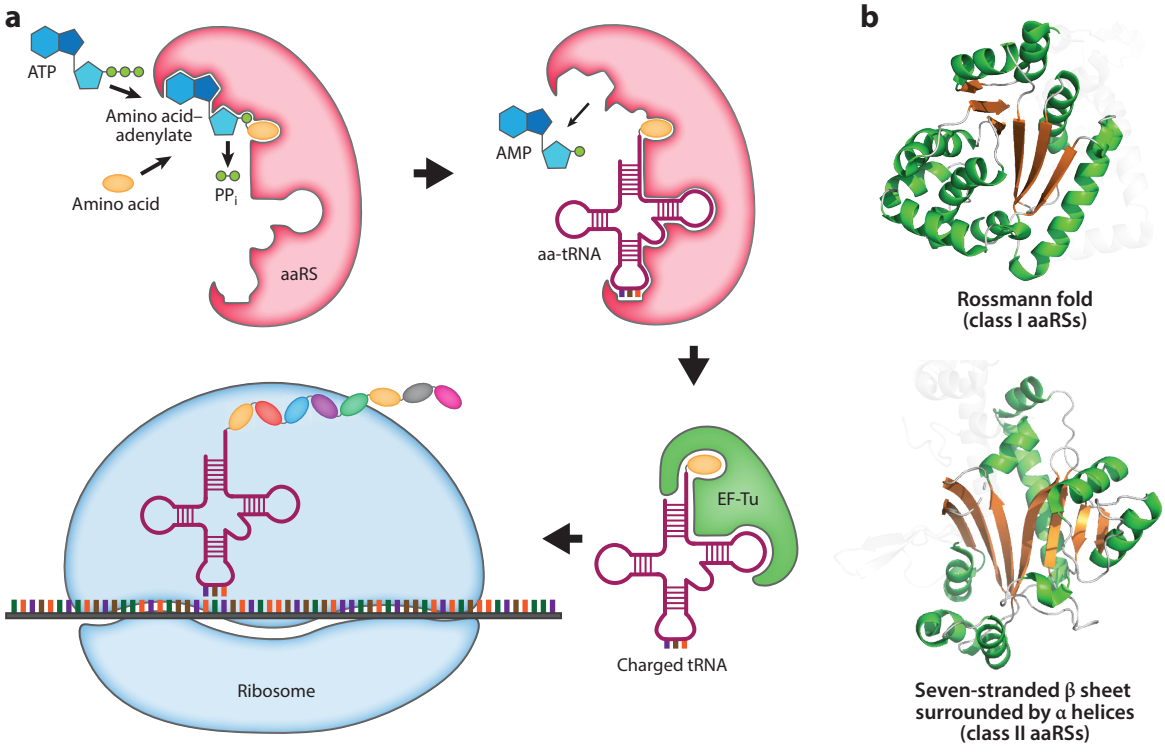

Figure 1

Diagram of tRNA charging by aaRSs and core structures of aaRSs. (*a*) aaRSs recognize a specific amino acid and use the energy from ATP to generate an activated adenylate intermediate. The cognate tRNA binds and is esterified to the amino acid, with the release of AMP. The charged tRNA is complexed to an elongation factor (EF-Tu) and delivered to the ribosome. The tRNA anticodon pairs with the cognate mRNA codon to ensure fidelity of protein synthesis. (*b*) The catalytic domain of class I aaRSs adopts a Rossmann fold (adapted from PDB 7ROU). The catalytic domain of class II aaRSs is characterized by a seven-stranded β sheet surrounded by α helices (adapted from PDB 5XIX). Abbreviations: aaRS, aminoacyl-tRNA synthetases; aa-tRNA, amino acid–tRNA; PDB, Protein Data Bank; PP_i, pyrophosphate.

each aaRS binds its cognate amino acid and ATP and forms an activated amino acid–adenylate with the release of pyrophosphate. In the second step the cognate tRNA binds, and the aaRS and the tRNA undergo conformational changes that position the tRNA acceptor stem to mount a nucleophilic attack on the amino acid–adenylate intermediate (29, 78). AMP is released and the aminoacyl tRNA (aa-tRNA) is delivered to the ribosome in complex with elongation factors (**Figure 1*a***). Considered the most ancient of enzymes, aaRSs are found in the cytoplasm of all bacteria, archaea, and eukaryotes, as well as in the mitochondria and plastids of eukaryotes. They comprise two distinct, apparently unrelated, superfamilies of enzymes (classes I and II) (16, 79) (**Figure 1*b***; see the sidebar titled Some Features of Class I and II Cytoplasmic aaRSs). Further classification into subclasses is based on sequence similarity and physicochemical properties of their amino acid substrates, and possibly reflects the origins of the two aaRS classes in ancestral protein-tRNA complexes (80).

SOME FEATURES OF CLASS I AND CLASS II CYTOPLASMIC aaRSs

Class I

Class I aaRSs are characterized by an N-terminal catalytic domain with a Rossmann fold structure linked to an α-helical C-terminal anticodon-binding domain that is involved in recognition of the tRNA.

Class Ia aaRSs charge methionine, valine, leucine, isoleucine, cysteine, and arginine. Class Ib aaRSs charge glutamic acid and glutamine (and lysine in archaea and a limited number of bacteria). Class Ic aaRSs charge tyrosine and tryptophan.

MetRS, CysRS, ArgRS, GluRS, and GlnRS have an editing domain connective peptide I that is expanded in ValRS, LeuRS, and IleRS and is repurposed as a dimerization domain in TyrRS and TrpRS. Class I aaRSs are generally monomeric.

Class I catalytic domains contain characteristic HIGH and KMSKS motifs (71). Formation of the adenylate intermediate is accompanied by a conformation change in the KMSKS loop (56).

TyrRS and TrpRS can have additional activities (e.g., signaling) that depend upon differential splicing events and generation of protein fragments (12, 100, 101).

Class I aaRSs generally bind to the minor groove of the acceptor stem of the tRNA and distort the tRNA 3′ terminus into a hairpin, allowing acylation of the 2′ OH group (15).

There are exceptions to the general rules: TyrRS, MetRS, and TrpRS are dimeric. TyrRS (32) and TrpRS (87, 106) bind tRNA in the major groove and can acylate the 3′ OH group of tRNA.

Class II

Class II aaRSs have a catalytic domain comprising a six-stranded, antiparallel β sheet flanked by α helices, linked to an anticodon or tRNA-binding domain. ProRS, ThrRS, and AlaRS contain editing domains.

Class IIa aaRSs charge serine, threonine, alanine, glycine, proline, and histidine. Class IIb aaRSs charge aspartic acid, asparagine, and lysine. Class IIc aaRSs charge phenylalanine.

Class II aaRSs are characterized by three conserved motifs involved in ATP binding and dimerization (42). They are generally dimeric or multimeric (48). Binding of substrates is associated with a conformational change.

In higher eukaryotes additional domains support noncanonical functions (e.g., signaling) (56). In general, class II aaRSs bind to the major groove of the tRNA in a manner that presents the 3′ OH of adenosine-76 at the 3′ end of the tRNA in position for attachment of the amino acid (25). As an exception, PheRS binds tRNA via the minor groove and attaches phenylalanine to the 2′ OH (107).

3. TARGETING PROTEIN TRANSLATION AS A PROMISING STRATEGY FOR DEVELOPMENT OF NEW ANTIMALARIALS

Protein translation is an essential pathway that is required at all stages of the malaria parasite's life cycle, making it a good target for the development of compounds with multistage activity. Antibiotics that inhibit protein translation by targeting the ribosome, such as tetracyclines, macrolides, and streptogramins, have been exploited successfully as antibacterial agents (31, 60, 98), and doxycycline is widely used for malaria prophylaxis (30). As critical components of the protein translation pathway, aaRSs are also promising targets for anti-infectives. Inhibition of an individual aaRS results in delivery of noncognate tRNAs, ribosome slipping, and synthesis of nonfunctional proteins, eventually leading to cell death (84).

aaRS enzymes have been targeted successfully in other pathogens. For example, mupirocin, an IleRS inhibitor, is a natural product that is widely used as a topical antibiotic (54). Agrocin 84 is a LeuRS inhibitor produced by a biocontrol agent, *Agrobacterium radiobacter*, that inhibits other pathogenic strains of agrobacteria (20). Halofuginone is a ProRS inhibitor used to prevent coccidiosis in poultry (76). Tavaborole is a LeuRS inhibitor used to treat fungal onychomycosis (34, 81, 89). GSK3036656, another LeuRS inhibitor, is undergoing trials in humans for treatment of tuberculosis (ClinicalTrials.gov NCT05382312) (94). These examples demonstrate proof-of-principle for aaRSs as therapeutic targets.

The *P. falciparum* nuclear genome has 37 aaRS genes encoding 36 aaRS enzymes that function in the cytoplasm and/or the apicoplast (11, 52). Charged tRNAs and one aaRS (PheRS) are also transported to the mitochondrion to support protein translation in that compartment (45). Phylogenetic analysis reveals that the *Plasmodium* cytoplasmic aaRSs cluster with other eukaryotic aaRSs and exhibit high-level conservation of active site residues, while apicoplast aaRSs cluster with bacterial aaRSs (11, 70).

Targeting apicoplast-located aaRSs appears, at first, to be a good strategy. However, apicoplast inhibitors result in a phenomenon known as delayed death (23), whereby parasite death occurs only in the second asexual cycle after treatment, when apicoplast-generated isoprenoid precursor metabolites become limiting (51). While apicoplast-targeting protein translation inhibitors, such as doxycycline, are used clinically for prophylaxis (30), their slow onset of action limits their usefulness for treatment of acute infections (58). Thus, this review concentrates on the challenge of identifying *Plasmodium* cytoplasmic aaRS inhibitors that achieve good potency while being sufficiently selective for the pathogen's enzyme over the equivalent host enzyme.

4. METHODS FOR IDENTIFYING AND CHARACTERIZING aaRS TARGETS AND INHIBITORS

4.1. Phenotypic Screening with In Vitro Evolution of Resistance and Genetic Validation

Based on their potent activity in other species, aaRSs have been proposed as antimalarial targets for many years. Unbiased phenotypic screens against the parasite asexual blood stage have identified several compounds that target *P. falciparum* aaRSs (3). These compounds were also shown to have activity in parasite liver stages (3). Such compounds are particularly attractive from a development point of view because they may also be used prophylactically.

In most cases, the association between a phenotypic screening hit and a particular aaRS is made using in vitro evolution of resistance (21). Clonal lines of *P. falciparum* in blood stages culture are incubated with the test compound for weeks to several months. Retrieved cultures that exhibit decreased susceptibility to the test compound likely harbor resistance-conferring mutations.

Whole-genome sequence analysis of multiple independently selected resistant clones reveals the newly emerged mutations (22). Such analyses were initially accomplished using tiling microarrays (LysRS, IleRS) but are now performed using whole-genome sequencing (61). Genetic lesions may include single-nucleotide polymorphisms (SNPs) or copy number variants. Independently selected clones often bear mutations in the same gene but with different allelic changes.

Only a few genetic changes emerge during the selection, and given that there are ~6,000 genes in the *P. falciparum* genome, the likelihood of a particular gene enrichment occurring by chance is small. For example, in clones derived from three separate selections with BRD1095, a bicyclic azetidine identified in a phenotypic screen, four independent mutations in PheRS were observed (49). The likelihood of these mutations arising by chance is less than 1 in 10^{50}. Nevertheless, further downstream validation is often needed and can help determine whether the gene of interest is the actual target or encodes a protein involved in multidrug resistance, such as an efflux protein. Studying the location of the mutation within the 3D structure of the enzyme reveals whether the mutation is near a predicted small-molecule binding site. Model organisms may also be used for evolution of resistance, often giving similar results. For example, evolution experiments with the *P. falciparum* LysRS inhibitor, cladosporin, in yeast yielded mutations in *Saccharomyces cerevisiae* LysRS (39).

To assess the contribution of a single allele to resistance, the mutations can be introduced into a clean genetic background using CRISPR-Cas9 gene editing methods (21). Similarly, conditional knockdown of the gene of interest is expected to enhance the potency of the inhibitor, whereas overexpression of the gene may decrease sensitivity (67, 72, 103). Genetic manipulation studies are also used to confirm that a particular *Plasmodium* aaRS is critical for survival of the parasite. The application of these methods is greatly facilitated by the Malaria Drug Accelerator (MalDA), a consortium of laboratories that was established to provide a target identification pipeline and to share resources and expertise (27, 105).

4.2. Biochemical Assays

In the first step of the aaRS-catalyzed reaction, ATP is consumed and pyrophosphate is released to generate the enzyme-bound aminoacyl-adenylate (**Figure 1*a***). Inorganic pyrophosphatase is added to prevent reversal of the reaction. The malachite green assay monitors the reaction of phosphate with molybdenum to form a colored complex (28, 47, 86, 108). The consumption of ATP can be monitored using assays such as the classical charcoal-absorption assay (10) or the Kinase-Glo assay, which monitors luciferase-catalyzed production of light (8, 39, 103).

In the second step of the aaRS-catalyzed reaction, the amino acid is conjugated to its corresponding tRNA, a reaction that can be monitored by trapping the charged radiolabeled amino acid onto filter discs (28). The cognate tRNA is usually generated by enzymatic synthesis using T7 RNA polymerase–mediated in vitro transcription (65), although commercially available preparations of mixed tRNAs can be used in some cases. Aminoacylation assays have been established for different plasmodium aaRSs (12, 85, 103).

Biochemical assays that monitor the first step of the reaction are favored in many laboratories, as they are simple to establish and can be adapted to higher-throughput formats; however, it should be noted that these assays will fail to identify inhibitors that inhibit the second step of the reaction. Moreover, some aaRSs require the presence of tRNA to catalyze the first reaction step (66).

4.3. Protein Translation Assays

Assays that measure protein translation in cells or in vitro are also valuable tools to assess the functional effects of inhibitors. Conventional protein translation assays monitor the incorporation

of radiolabeled amino acids into proteins in whole cells (5, 13, 88). More recently an assay that monitors the incorporation of a fluorescent puromycin derivative, *O*-propargyl-puromycin (OPP) (96), has been adapted to *P. falciparum* with a flow cytometric readout (24, 103).

In addition, different laboratories have developed methods for preparing translation-competent lysates of *P. falciparum* and measuring translation in vitro with luciferase reporters (9, 88, 93). The quality of the lysate is a variable that can complicate these assays, but they have the advantage of being suitable for adaption to higher-throughput formats; and the corresponding human cell lysates can be assayed in parallel to identify selective inhibitors of plasmodium translation (93).

4.4. Binding Assays

Thermal stabilization assays involve heat-mediated denaturation of the protein of interest using a thermal cycler (41). Differential scanning fluorimetry (DSF) is used to measure the signal from a dye, such as SYPRO Orange, that binds to hydrophobic regions that become exposed as the protein denatures. Tight-binding ligands increase the apparent protein melting point, which can provide an estimate of the inhibitor binding affinity (6). DSF has been used to measure binding of inhibitors to aaRSs (8, 37, 53, 103, 109). An alternative time-resolved Förster resonance energy transfer assay was recently developed that can be used to characterize binding of ligands to ProRS (95).

4.5. Analysis of the Amino Acid Starvation Response

Accumulation of uncharged tRNAs triggers the amino acid starvation stress response (17). The anticodon binding domain in the C-terminal region of the *P. falciparum* GCN2 homolog, *Pf*eIK1, mediates binding to uncharged tRNA, which in turn leads to eIF2α phosphorylation (26) and stalling of protein synthesis (5). Thus, eIF2α phosphorylation offers a useful diagnostic tool to validate aaRS targets (35, 43, 103).

5. TARGETING *PLASMODIUM* aaRSs

Plasmodium aaRSs are suitable targets for the development of new antimalarials, and several previous reviews have covered different aspects of efforts to target these enzymes (19, 52, 63, 70, 75, 78). Here, we focus on inhibitors of four cytoplasmic aaRSs that have shown efficacy in a humanized mouse model of *P. falciparum* malaria and where structural analysis has informed our understanding of the mode of action. Compounds that target other *Plasmodium* aaRS have also been investigated and we refer the reader to studies of cytoplasmic LeuRS (89), MetRS (40), ThrRS (69, 91), IleRS (43, 44), and apicoplast IleRS (38).

5.1. *Plasmodium falciparum* LysRS

A screen of a natural product library identified a fungal secondary metabolite, the isocoumarin analogue cladosporin (**Figure 2**), possessing activity against blood- and liver-stage *P. falciparum* in cell culture assays (39). The activity against 3D7 cultures ($IC_{50} = 45$ nM) was 970-fold more potent than against the mammalian HepG2 cell line. A yeast haploinsufficiency study and in vitro evolution of resistance studies in *P. falciparum* and *S. cerevisiae* each pointed to cytoplasmic *Pf*LysRS, a Class II aaRS (**Table 1**), as the target. The target was confirmed by demonstrating that cladosporin inhibits protein translation in *P. falciparum* and that overexpression of *Pf*LysRS decreased sensitivity to the inhibitor (39). Biochemical assays using recombinant *Pf*LysRS and human (*Hs*) LysRS confirmed the selectivity; and provided evidence that cladosporin interacts with the ATP-binding pocket of *Pf*LysRS (39, 53).

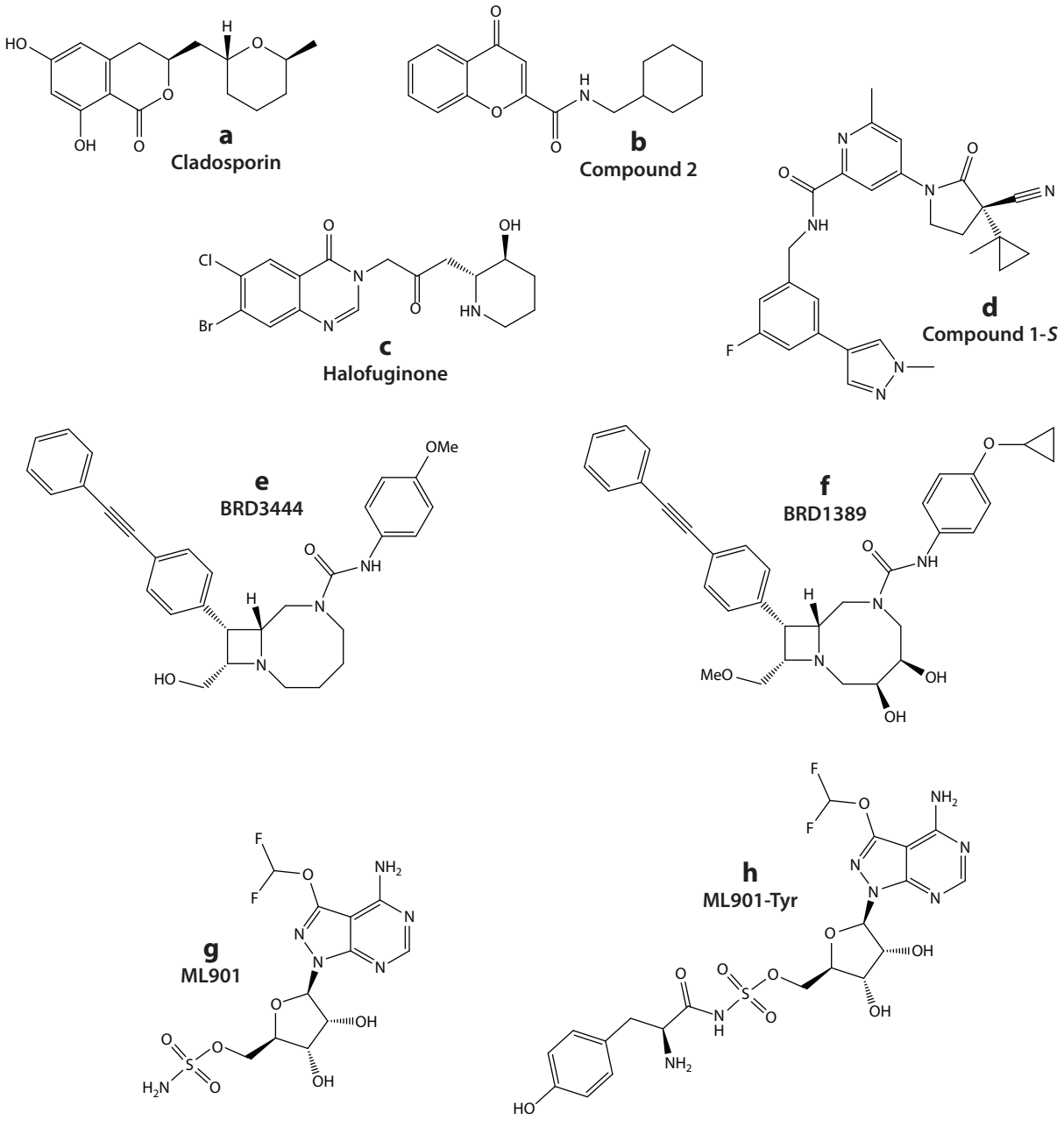

Figure 2

Structures of some aminoacyl-tRNA synthetase inhibitors: (*a*) cladosporin, (*b*) difluoro cyclohexyl chromone (compound 2), (*c*) halofuginone, (*d*) 1-(pyridin-4-yl) pyrrolidin-2-one derivative (compound 1-*S*). (*e*) BRD3444, (*f*) BRD1389, (*g*) ML901, (*h*) ML901-Tyr.

Table 1 Features of selected *Plasmodium falciparum* cytoplasmic aaRSs

aaRS	Class	Structural features	2′/3′ OH amino-acylated	tRNA groove bound	Inhibitors	Binding pocket occupied
LysRS	IIb	Dimer. ABD, α/β catalytic domain, motifs I–III	3′	Major	Cladosporin/ chromene-2-carboxamides	ATP
ProRS	IIa	Dimer. α/β catalytic domain, motifs I–III, ABD, Z-domain	3′	Major	Febrifugine/ halofuginone/ 1-(pyridin-4-yl) pyrrolidin-2-ones	Proline/$tRNA^{Pro}$-A76
PheRS	IIc	Tetramer. TBD, $(\alpha/\beta)_2$ (α-1 catalytic, β-3/4 editing), motifs I–III	3′	Major	Bicyclic azetidines	Phe + ATP
TyrRS	Ic	Dimer. Rossmann fold catalytic domain, dimerization domain, ABD	2′ and 3′	Major	ML901-Tyr/ AMS-Tyr (formed in situ)	Tyr + ATP

Abbreviations: aaRS, aminoacyl-tRNA synthetase; ABD, anticodon-binding domain; AMS, adenosine 5′ sulfamate; TBD, tRNA-binding domain.

Structural studies showed that cladosporin adopts an AMP mimicking conformation, with the isocoumarin moiety binding in the adenine pocket and the 2,6-disubstituted tetrahydropyran accommodated in the ribose binding pocket of *Pf*LysRS (53). Comparison of this structure with that of *Hs*LysRS in complex with Lys and ATP (33) showed that *Pf*LysRS Ser344 adopts a rotamer conformation that enhances cladosporin binding, while a steric clash with the equivalent *Hs*LysRS Thr337 is expected to prevent binding (53). Interestingly, ligand-induced fit of cladosporin into *Pf*LysRS but not *Hs*LysRS also appears to contribute to the binding specificity (18), suggesting that selectivity can involve dynamic effects as well as differences in the amino acids directly lining the active sites.

The metabolic instability and poor oral bioavailability of cladosporin preclude further development of this compound; and attempts have been made to generate cladosporin derivatives (83, 109), to move to different scaffolds (4), and to repurpose inhibitors from other indications (108). Another approach has been to screen a library of small molecular weight molecules, using the Kinase-Glo assay, with a counter-screen against *Hs*LysRS, leading to the discovery of compound 2 (**Figure 2**), a chromene-2-carboxamide with potent activity against *Pf*LysRS but poor metabolic stability (8). Addition of fluorine and hydroxyl substituents to the cyclohexyl ring structure to generate compound 5 enhanced the metabolic stability and provided excellent oral bioavailability (F = 100%) (8). Compound 5 is active against liver schizonts and shows a good in vitro selectivity profile, although the half-life (in mice) remains moderate ($T_{1/2}$ = 2.5 h); and the compound exhibits a slow rate of killing. In a SCID mouse model of *P. falciparum* malaria, compound 5 reduced parasitemia by 90% after four daily doses of 20 or 40 mg/ml. However, toxicity was observed in mice at higher doses (50 mg/kg orally).

A crystal structure of compound 2 in complex with *Pf*LysRS and Lys revealed that the chromone core binds in the adenine binding site, while the cyclohexyl ring occupies the ribose binding pocket (**Figure 3*a***). Molecular dynamics simulations and thermal shift assays suggest that ligand-induced stabilization of a loop and other residues near the *Pf*LysRS active site could potentially underlie the favorable binding of chromene-2-carboxamides to *Pf*LysRS compared with *Hs*LysRS (**Figure 3*b***).

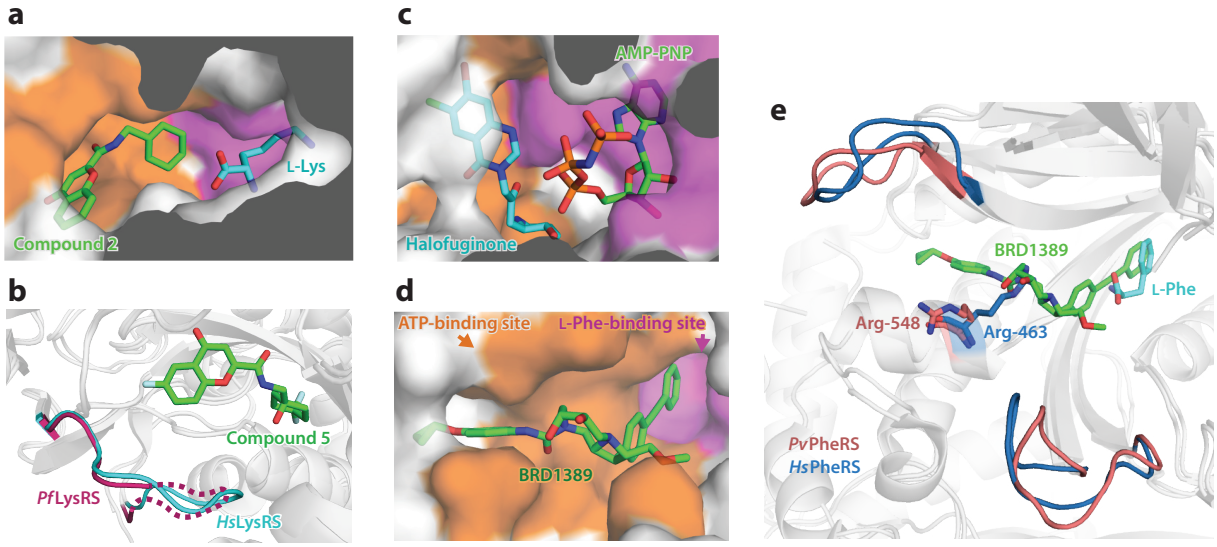

Figure 3

Views of the binding sites of cytoplasmic *P. falciparum* LysRS (*Pf*LysRS), *Pf*ProRS, and *Pf*PheRS illustrating inhibitor binding modes. (*a*) Structure of *Pf*LysRS in complex with compound 2 (*green*) and L-lysine (*cyan*) (PDB 6AGT). The compound 2 binding site is shown in orange and the L-lysine binding site is shown in magenta. (*b*) Overlay of compound 5 (a compound 2 derivative) bound to *Pf*LysRS (*magenta*) (PDB 6HCU) and human LysRS (*Hs*LysRS) (*cyan*) (PDB 4YCU). An active-site loop is highly stable in *Hs*LysRS, while the equivalent loop (*dashed line*) in *Pf*LysRS is mobile but becomes partially stabilized upon ligand binding. (*c*) Structure of *Pf*ProRS in complex with halofuginone (*cyan*) and AMP-PNP (*green*) (PDB 4YDQ). Halofuginone- and AMP-PNP-binding pockets are shown in orange and magenta, respectively. (*d*) Structure of *P. vivax* PheRS (*Pv*PheRS) with bound BRD1389 (PDB 7BY6). The L-phenylalanine-binding pocket and ATP-binding site are indicated by arrows. (*e*) Overlay of BRD1389-bound *Pv*PheRS (*pink*) and L-phenylalanine-bound *Hs*PheRS (*blue*) (PDB 3L4G). The open conformation of Arg-548 in *Pv*PheRS allows the binding of BRD1389, while the equivalent Arg-463 in *Hs*PheRS adopts a closed conformation that causes a steric clash. Different conformations of two loops adjacent to the active sites of *Pv*PheRS and *Hs*PheRS may also contribute to the selectivity for the *Plasmodium* enzyme. Abbreviations: AMP-PNP, adenylyl-imidodiphosphate; PDB, Protein Data Bank.

5.2. *Plasmodium falciparum* ProRS

Extracts from the roots of the blue evergreen hydrangea, *Dichroa febrifuga*, were used in traditional Chinese medicine to treat malaria fevers (55, 59). The active ingredient, the quinazolinone alkaloid febrifugine, is a potent inhibitor of the class II aaRS, ProRS (50) (**Table 1**). The synthetic halogenated derivative, halofuginone (**Figure 2**), is used in veterinary medicine to treat coccidia (68).

While febrifugine and halofuginone exhibit unwanted side effects and are not considered suitable for development as antimalarials, derivatives have been identified that exhibit better specificity, while maintaining potency against *Plasmodium* (35, 46, 50, 92). Structural and biochemical analyses revealed that halofuginone and derivatives exert their activity through competitive binding into the proline site of cytoplasmic *Pf*ProRS (**Figure 3*c***) (35, 46, 50). The proline site is highly conserved between the plasmodium and human enzymes; although there is a disordered loop that folds over the binding site that may contribute to differential binding (46). The binding mode leaves these inhibitors susceptible to resistance via upregulation of proline accumulation by *P. falciparum*, as well as via amplification of the *Pf*ProRS locus and SNPs in the *Pf*ProRS gene (35, 36).

To improve selectivity, 40,000 compounds were screened from five compound libraries (SPECTRUM Microsource, GlaxoSmithKline Tres Cantos Antimalarial Set, the DDU small

diversity set, Malaria Box and the St. Jude Library) to identify compounds that selectively inhibit *Pf*ProRS enzyme activity with a counter screen against *Hs*ProRS (37). Two compounds were identified, glyburide and TCMDC-124506, that exhibited good specificity, though lower activity than halofuginone. Interestingly, these compounds bind in a pocket adjacent to the ATP binding site.

Takeda Pharmaceuticals screened 500,000 compounds in a biochemical assay and identified novel pyrazinamides as *Hs*ProRS inhibitors that bind competitively in the ATP site (1). Phenotypic screening of about 200 of these inhibitors against *P. falciparum* cultures identified a 1-(pyridin-4-yl)pyrrolidin-2-one derivative (compound 1-*S*, **Figure 2**) that showed good potency (IC_{50} ~10 nM). Compound 1-*S* showed 36-fold selectivity in a biochemical assay compared with *Hs*ProRS, and 77-fold selectivity compared with a mammalian cell line (72). Compound 1-*S* demonstrated oral efficacy with 4 daily doses at 50 mg/kg in a SCID mouse model of *P. falciparum* malaria. As an ATP-competitive inhibitor, off-target activity is a potential liability and activity against adenosine A3 kinase was identified as a potential concern. The series has liabilities with respect to a relatively slow killing profile and a propensity for development of resistance, due to ready emergence of copy number variations in the *Pf*ProRS gene locus.

An interesting approach to increase the potency is to combine two inhibitors that bind at different sites. A recent study targeted ProRS from *Toxoplasma gondii*, a related apicomplexan parasite, with two inhibitors, halofuginone and a novel pyrrolidine-based ATP mimetic (L95). The authors used X-ray crystallography to show that the inhibitors occupy all three of the enzyme subsites (64). Similarly, a hybrid compound was generated comprising a piperidyl pyrazinamide, NCP26, which binds in the adenylate site, coupled via an acylphosphate-mimicking sulfamoylcarbamate, to halofuginone, which binds across the $tRNA^{Pro}$-A76 and proline pockets. The compound engages all three substrate-binding pockets in *Pf*ProRS; and binds with high affinity (95).

5.3. *Plasmodium falciparum* PheRS

Phenotypic screening of a Broad Institute library of ~100,000 compounds generated via diversity-oriented synthesis identified a series of novel antimalarial bicyclic azetidines (exemplar BRD3444, **Figure 2**) with activity against asexual parasites and late stage gametocytes, as well as against liver stage parasites (49). In vitro evolution of resistance yielded clones with SNPs close to the Phe binding site in the α subunit of cytoplasmic *Pf*PheRS. PheRS is a class II heterodimer, comprising a catalytic α subunit and a tRNA anticodon-binding (β) subunit, that further dimerizes to form an $(\alpha\beta)_2$ tetramer (32) (**Table 1**). BRD3444 showed potent inhibition of aminoacylation of the cognate *Pf*$tRNA^{Phe}$ by recombinant *Pf*PheRS (IC_{50} = 46 nM). L-Phe decreased the potency of BRD3444 indicating that it binds in the Phe-binding site.

BRD3444 exhibited poor solubility and suboptimal pharmacokinetic properties, including a short half-life in vivo. Replacement of the hydroxymethyl group at position C2 on the azetidine ring with an aminomethyl group yielded BRD7929, which exhibited improved potency in vitro and a longer half-life in vivo (32 h). BRD7929 showed efficacy as a single oral dose (25 or 50 mg/kg) in a SCID mouse model of *P. falciparum* malaria (49). BRD7929 was also effective at eliminating liver-stage parasites in two different models and in preventing parasite transmission to mosquitoes (49). No recrudescence was observed when cultures containing 10^9 parasites were exposed to a constant pressure with BRD7929, indicating a very high Minimum Inoculum for Resistance (MIR). A limitation with BRD7929 was moderate cytotoxicity against a human cell line (HepG2 IC_{50} = 9 μM) and hERG inhibition. Moreover, the complex synthetic route to the bicyclic azetidines makes it difficult to achieve a low cost of goods. An alternative synthetic route yielded BRD3914, with improved potency and selectivity, but less favorable pharmacokinetic properties. Nonetheless, BRD3914 is effective in the SCID mouse model after four oral doses (62).

An X-ray crystal structure of *P. vivax* PheRS (*Pv*PheRS) in complex with a cyclopropoxy bicyclic azetidine ligand (BRD1389) (**Figure 2**) revealed that the inhibitor occupies the Phe-binding site but also extends into a cavity that lies adjacent to the ATP binding site (**Figure 3*d***). A comparison of the *Pv*PheRS and *Hs*PheRS-Phe complexes provides evidence that Arg548 flexes to permit induced fit binding into *Pv*PheRS (**Figure 3*d***). Similarly, differential flexibility of two loops adjacent to the active site may contribute to the selectivity for the *Plasmodium* enzyme (86) (**Figure 3*e***).

It is worth noting that related bicyclic azetidines have shown in vivo efficacy against other protist pathogens, including against the diarrheal pathogen *Cryptosporidium parvum* (99) and against both acute and chronic stages of *T. gondii* infection (77). This finding provides support for the suggestion that aaRS inhibitors may exhibit efficacy across different protist pathogens.

5.4. *Plasmodium falciparum* TyrRS and Reaction Hijacking Pro-Inhibitors—A New Avenue for Targeting aaRSs

A recent study identified an important new compound that specifically targets a *P. falciparum* aaRS with a novel mechanism of action (103). A phenotypic screen of a Takeda Pharmaceutical library of about 2,000 nucleoside sulfamates identified pyrazolopyrimidine sulfamates (exemplar ML901, **Figure 2**) that exhibit good potency against *P. falciparum* cultures (3D7 IC_{50} < 10 nM) and over 1,000-fold selectivity compared with mammalian cell lines. ML901 exerts potent activity against liver schizonts and male gametes, consistent with whole-of-life-cycle killing. It exhibits a long terminal half-life in blood (41 h) following intravenous or oral dosing in rats. It exhibits single-dose efficacy (50 mg/kg i.p.) in a mouse model of malaria. The pyrazolopyrimidine sulfamate chemotype represents a starting point for the development of compounds that maintain potency and favorable half-life while exhibiting even higher specificity for plasmodium and better oral bioavailability (103).

The target of ML901 was identified using in vitro evolution of resistance, as cytoplasmic *P. falciparum* TyrRS, a class I aaRS (103) (**Table 1**). Biochemical studies revealed that *Pf* TyrRS is susceptible to an unusual inhibition mechanism, referred to as reaction hijacking. The data suggest that ML901 binds to the AMP-vacated site on the enzyme while the product, Tyr-tRNA^{Tyr}, remains bound and that it mounts a nucleophilic attack on the charged tRNA (**Figure 4*a***),

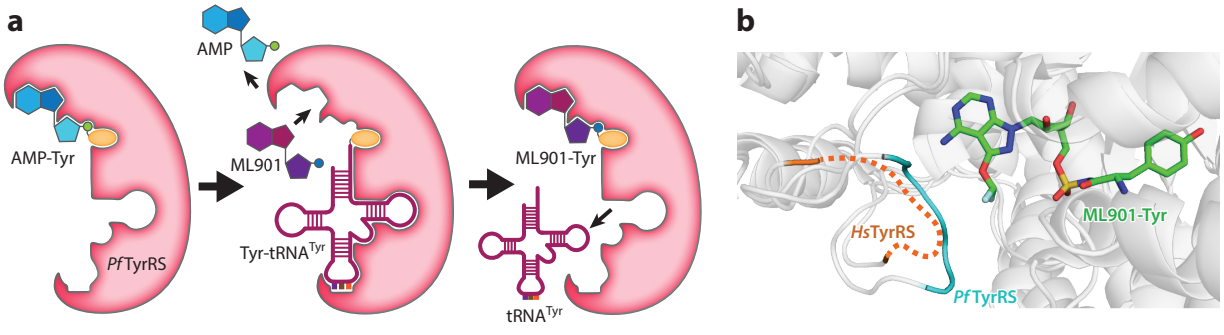

Figure 4

Model and structural analysis of TyrRSs revealing the molecular basis for susceptibility to reaction hijacking. (*a*) Schematic of the reaction hijacking mechanism. ML901 mounts a nucleophilic attack on the enzyme-bound charged tRNA to generate the ML901-Tyr inhibitor. (*b*) Overlay of *P. falciparum* TyrRS (*Pf* TyrRS) (*cyan*) (PDB 7ROS) and human TyrRS (*Hs*TyrRS) (*orange*) (PDB 7ROU) in complex with ML901-Tyr. The KMSKS loop motif of *Pf* TyrRS forms a flap over the active site, while the equivalent loop in *Hs*TyrRS (*dashed line*) is mobile. This difference appears to underpin the differential susceptibility to reaction hijacking by ML901. Abbreviations: PDB, Protein Data Bank; TyrRS, tyrosine tRNA synthetase.

generating a Tyr-ML901 adduct (**Figure 2**). Targeted mass spectrometry was used to detect the signature Tyr-ML901 covalent adduct in parasites treated with ML901 (103). The mechanism is both surprising and exciting. ML901 is a small-molecular-weight, membrane-permeable compound that acts as a proinhibitor. That is, it is converted within the active site to a tight-binding inhibitor that occupies both the adenosine-binding pockets and the amino acid–binding pocket (103).

Xie et al. (103) confirmed that ML901 inhibits protein translation in an in-cell assay. ML901 exposure leads to inhibition of ATP consumption by recombinant *Pf* TyrRS—but only in the presence of all substrates, i.e., tyrosine, ATP, and $tRNA^{Tyr}$, consistent with a reaction hijacking mechanism. X-ray crystallographic studies of plasmodium and human TyrRSs reveal differential mobility of the KMSKS loop over the catalytic site (**Figure 4*b***). This difference appears to underpin the differential susceptibility to reaction hijacking by ML901. The KMSKS motif is conserved across apicomplexan and kinetoplastid parasites, suggesting potential for cross pathogen activity.

Adenosine 5′ sulfamate (AMS) was also identified as a broad-specificity proinhibitor of *P. falciparum* cultures ($IC_{50} = 2$ nM). Mass spectrometry of AMS-treated parasites revealed that it induces formation of a range of amino acid–AMS adducts, consistent with reaction hijacking of the class I aaRS TyrRS and several class II aaRSs: AsnRS, AspRS, SerRS, ThrRS, GlyRS, AlaRS, LysRS, and ProRS (103). The cross class activity of AMS is intriguing, and in this context, it is interesting to note that TyrRS is an atypical class I aaRS. It resembles class II aaRSs in that it is dimeric, it binds tRNA in the major groove, and it can catalyze aminoacylation of the 3′ OH of the 3′ adenosine-76 (**Table 1**).

The potency of AMS as a proinhibitor is consistent with previous studies showing that synthetic amino acid–AMS conjugates exhibit potent activity against *P. falciparum* (69). AMS is not suitable for further development as an antimalarial due to its broad reactivity, including reaction hijacking of human aaRSs (103). Nonetheless, the data indicate that nucleoside sulfamate libraries could prove a very exciting source of bespoke compounds that target *Plasmodium* but not human aaRSs, leading to new antimalarial candidates. Indeed, given the importance of aaRSs in all organisms, bespoke nucleoside sulfamates may find applications in a broad range of infectious and metabolic diseases.

6. CONCLUSION

The *P. falciparum* aaRSs are essential for malaria parasites at all stages of growth; and accordingly, aaRS inhibitors exhibit potent whole-of-life-cycle activity against *P. falciparum*, making aaRSs desirable targets. The cytoplasmic aaRSs are preferred targets compared with apicoplast-located aaRSs due to the delayed death phenotype associated with the latter targets. *P. falciparum* requires 20 cytoplasmic aaRSs, each with at least three potentially targetable substrate-binding sites. All aaRSs catalyze the same general two-step reaction: adenylating an amino acid and then conjugating it to its cognate tRNA. Nonetheless, structural divergence of *Pf* aaRSs from their human homologs offers many possible sites for inhibition. By contrast, individual aaRSs are very well-conserved across different plasmodium species and other protist parasites, offering the possibility of developing cross pathogen inhibitors. Human cells may also be less sensitive to partial inhibition of aaRSs under physiological conditions because the normal levels of aaRS activity are well above what is needed to sustain translation (57, 104).

Recent years have seen the development of efficient tools for identifying different *Pf* aaRS targets. Phenotypic screens of inhibitor libraries against parasite cultures have provided multiple hits, while counter-screens against mammalian cell lines and streamlined biochemical and biophysical assays are available to validate the target, assess selectivity, and determine whether the inhibitors have drug-like properties.

Structural studies have been invaluable for understanding the molecular basis for the specificity and potency of different aaRS inhibitors. While the active sites are often well conserved, subtle differences can be exploited to enable differential inhibition. A common theme that is emerging is that flexible residues or loops near the active site, coupled with ligand-induced conformational changes, can underpin differential susceptibilities of *Plasmodium* and human enzymes. Such conformational changes are difficult to predict, illustrating the need for ongoing structural biology studies to accompany medicinal chemistry efforts to improve potency and selectivity.

There are potential liabilities associated with aaRS-targeting compounds. For example, it is clear that *P. falciparum* can acquire resistance to aaRS inhibitors. Inhibitors that bind only at the amino acid–binding site may be susceptible to upregulation of amino acid accumulation (36), while SNPs that inhibit binding at other pockets can be selected (35, 49, 72, 103). However, some aaRS-targeted inhibitors show a low propensity for the selection of resistance using a standardized protocol (49), indicating that compounds with suitable characteristics can be achieved. Inhibitors that target two or more aaRSs or bind tightly across the adjacent amino acid– and adenylate-binding sites may be more refractory to the development of resistance.

Off-target activity is another potential liability, particularly for inhibitors that bind at the adenosine-binding site. Inhibitors, such as the bicyclic azetidines and the ML901-Tyr adduct, which bind across two binding sites, may avoid these issues. Compounds that bind across two sites may also have increased binding affinity, and thus potency, though the complexity of synthetic routes and poorer drug-like properties may be a disadvantage.

Looking ahead, the advent of nucleoside sulfamate proinhibitors is an exciting new opportunity to generate inhibitors that target two binding pockets in situ. The proinhibitor mechanism expands opportunities to achieve good oral bioavailability. It is also very exciting that a boron-based aaRS suicide inhibitor (tavaborole) that forms a stable adduct with $tRNA^{Leu}$, trapping the enzyme-bound tRNA in the editing site, has been approved by the US Food and Drug Administration as an antifungal agent (81). If a similar benzoxaborole inhibitor of *Pf*LeuRS could be identified, this would provide new avenues for development of dual-pocket-binding antimalarials.

In the absence of broadly effective vaccines, and in the face of emerging resistance to current antimalarials, there remains an urgent need to develop new drug candidates. MMV criteria for advancing antimalarial candidates are very stringent; and currently, no aaRS inhibitor has been selected as a preclinical candidate. Nonetheless, given their potential to deliver potent and selective single-dose treatment for uncomplicated malaria, across different strains and species, as well as their potential for chemoprophylaxis and transmission blocking, aaRS inhibitors seem likely to feature as next-generation antimalarials.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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