Inhibition of Protein Synthesis and Malaria Parasite Development by Drug Targeting of Methionyl-tRNA Synthetases

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Aminoacyl-tRNA synthetases (aaRSs) are housekeeping enzymes that couple cognate tRNAs with amino acids to transmit genomic information for protein translation. The Plasmodium falciparum nuclear genome encodes two P. falciparum methionyl-tRNA synthetases (PfMRS), termed PfMRS<sup>cyt</sup> and PfMRS<sup>api</sup>. Phylogenetic analyses revealed that the two proteins are of primitive origin and are related to heterokonts (PfMRS<sup>cyt</sup>) or proteobacteria/primitive bacteria (PfMRS<sup>api</sup>). We show that PfMRS<sup>cyt</sup> localizes in parasite cytoplasm, while PfMRS<sup>api</sup> localizes to apicoplasts in asexual stages of malaria parasites. Two known bacterial MRS inhibitors, REP3123 and REP8839, hampered Plasmodium growth very effectively in the early and late stages of parasite development. Small-molecule drug-like libraries were screened against modeled PfMRS structures, and several “hit” compounds showed significant effects on parasite growth. We then tested the effects of the hit compounds on protein translation by labeling nascent proteins with<sup>35</sup>S-labeled cysteine and methionine. Three of the tested compounds reduced protein synthesis and also blocked parasite growth progression from the ring stage to the trophozoite stage. Drug docking studies suggested distinct modes of binding for the three compounds, compared with the enzyme product methionyl adenylate. Therefore, this study provides new targets (PfMRSs) and hit compounds that can be explored for development as antimalarial drugs.
Malaria parasite aaRSs are currently being explored as new targets for drug development (22, 23). Within aaRSs, MRSs can serve as valuable drug targets because of their sequence and domain heterogeneity. Inhibitors that target MRSs are already under development against bacterial infections (24). Derivatives of diarylamines, quinolones, urea, and various other lead compounds with potent activities against MRSs have been tested (25–27). Therefore, we decided to explore various attributes of malarial MRSs with the aim of probing their potential for drug targeting. Here we report the localization and phylogenetic analysis of both copies of PfMRSs. We also provide parasite growth inhibition data using drug-like compounds to address the feasibility of targeting PfMRSs. Some of the “hit” compounds are able to abrogate protein translation in malaria parasites, suggesting that they likely target the active sites of PfMRSs. In summary, our data add to the growing family of parasite aaRSs that can be targeted for inhibitor development against malaria parasites.

MATERIALS AND METHODS

Cloning, expression, and purification of subdomains of PfMRSs and antibody generation. Clones of the N- and C-terminal domains of MRSV and the anticond binding domain of MRSW were synthesized from full-length genes using the following primer pairs: (i) forward, GC TCCATGGGAATTCATGATG; reverse, GTGTAACCTTATTTAATTATG GCCTGGTGATGATAA; (ii) forward, GCTCAATGGGCCGCAAAAT TAAACTGCAG; reverse, GTGTAACCTTATTTAATTATG GCCTGGTGATGATAA; (iii) forward, GCTCATTGGAAATGAGCGAATCATCAG; reverse, TGGTACCTTATTTAATTATG GCCTGGTGATGATAA. The vector PetM11 was used for protein expression in E. coli (BL21 (DE3)). Culture medium for growing transformed cells was inoculated with 1% culture grown overnight at 37°C until the optical density (OD) at 600 nm reached 0.8. Protein expression was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 18°C, and cells were allowed to grow for 10 to 12 h. The cells were harvested at 5,000 × g for 30 min and sonicated, and proteins were purified using immobilized metal affinity chromatography. A further purification step of gel permeation chromatography and ion-exchange chromatography was carried out to purify target proteins. Antibodies against PfMRSs were generated in rabbits, and previously characterized antibodies against parasite proteins were used as controls where appropriate (28, 29).

Culture of Plasmodium falciparum 3D7 and D10-ACP leader-GFP-transfected cells. Plasmodium falciparum 3D7 cells were cultured with O2 red blood cells (RBCs) in RPMI 1640 medium (Invitrogen) supplemented with 4.5 mg mL−1 glycine (Sigma), 0.1 mM hypoxanthine (Invitrogen), 25 mg mL−1 gentamycin (Invitrogen), and 0.5% AlbuMax 1 (Invitrogen), according to standard methods. Parasites were treated with sorbitol in the ring stage to maintain synchronized cultures, as described previously (30). The Plasmodium falciparum D10-acyl carrier protein (ACP) leader-green fluorescent protein (GFP) transflectant line, in which GFP is targeted to the apicoplast by the leader peptide of ACP, was cultured similarly and supplemented with the addition of pyrimethamine (10 μg mL−1), and then the coverslips were washed three times with PBS, mounted in antifade medium with 4′,6-diamidino-2-phenylindole (DAPI), and sealed. Confocal microscopy was performed with immunofluorescently labeled parasites by using a Nikon A1R microscope with diode (405 nm), argon (488 nm), and helium-neon green (543 nm) lasers, with a 100× oil immersion lens. Images were viewed and analyzed using NIS-Elements software (version 3.2).

Modeling, in silico screening, and phylogenetic analyses. For modeling of the synthetase domains of PfMRSs, the sequences were compared with homologs of known structure through BLAST analysis with the Protein Data Bank (PDB). The MRS from Thermus thermophilus (PDB accession number 1A8H) was found to be the nearest homolog and was used as the template. Prior to modeling, low-complexity regions (LCRs) in the PfMRSs were removed. Atomic models were obtained using Modeller (31) and were selected based on discrete optimized protein energy (DOPE) and modular objective function (MOF) scores. Subsequently, models were validated using the structural analysis verification server (SAVES) (http://nshserver.mbi.ucla.edu/SAVES). PfMRS structural models were subjected to energy minimization using the Prime module in the Schrödinger suite (Prime version 3.0; Schrödinger, LLC, New York, NY), Ramachandran plot outliers were fixed, and models were revalidated using SAVES. The active sites of the PfMRS three-dimensional models were analyzed using Chimera (32), in the context of sequence alignments and conserved motif information. The small-molecule drug-like library (~50,000 compounds) was downloaded from Specs (http://www.specs.net). The library was prepared using the LigPrep and QikProp modules in the Schrödinger suite and was passed through 10 lead-like filters and analysis with Lipinski’s rule of five. The PfMRSV model was prepared using the PrepWizard module and was used to build an energy grid. For Glide docking, the box was centered on active site residues identified from methionyl adenylate (MOD)-bound MRS complex structures. The Glide extra precision (XP) algorithm was used to perform virtual screening. The top-ranking ligands were selected based on docking scores and H-bond interactions. Interactions of potential inhibitors were analyzed using Chimera (32) and LigPlot (33). The average logp and logS values were calculated using the Virtual Computational Chemistry Laboratory server.

FIG 1 Overall domain architecture of MRSs from prokaryotes and eukaryotes. The PfMRS proteins bear additional domains at the N and C termini, with no significant homology to other MRSs. HsMRS, Homo sapiens MRS; EcMRS, E. coli MRS; ScMRS, Saccharomyces cerevisiae MRS.
For phylogenetic analysis, 61 MRS sequences were aligned by using ClustalW2 and a phylogenetic tree was constructed with the maximum likelihood method by using the Mega 5.0 tool (34, 35).

In vitro parasite inhibition assays and 35S labeling of Plasmodium falciparum. The Plasmodium falciparum 3D7 strain was cultured in 96-well plates and synchronized at the ring stage using sorbitol. At 1% parasitemia and 2% hematocrit levels, the compounds were incubated for 48 h with parasite cultures, and the mixtures were assayed by the SYBR green I DNA staining method, as described previously (36, 37). After 48 h, 100 μl SYBR green dye at 1× concentration in lysis buffer supplemented with 0.1% saponin was added to each well. After 45 min of incubation at 37°C, fluorescence was estimated using a multiwell plate reader (Victor3; PerkinElmer), with excitation and emission wavelength bands centered at 485 and 530 nm, respectively. Anisomycin and pyrimethamine were used as control compounds, and all experiments were performed in triplicate. The 50% effective concentration (EC_{50}) values were obtained by plotting fluorescence values, expressed in terms of percent inhibition of parasite growth, at each inhibitor concentration. For protein synthesis inhibition assays, asynchronous cultures of the Plasmodium falciparum 3D7 strain were harvested at parasitemia levels of ~7 to 10% and hematocrit levels of 4%. The infected RBCs (iRBCs) were washed three times with methionine- and cysteine-free medium (Sigma) and were aliquoted in triplicate into 96-well plates. The selected compounds were added to culture plates in serially diluted concentrations from 0.1 nM to 100 μM. Anisomycin (Sigma), a known inhibitor of protein translation, was used as a positive control. The remaining steps were performed as reported previously (38). Counts were measured in a scintillation counter (Beckman Coulter) and compared with those of precipitates of parasites not treated with inhibitor or compounds. Plots were prepared with GraphPad Prism 6 software.

Growth inhibition by REP3123 and REP8839. The maturation of schizonts was evaluated by incubating iRBCs, at 0.5 to 1% parasitemia, in the presence of REP3123 or REP8839. Parasites in the later stages of development (~48 h) were treated with inhibitors at 200 nM or 500 nM. The final dimethyl sulfoxide (DMSO) concentration was maintained below 1%. The maturation of schizonts was counted on thick-smear glass slides after 24 h of treatment. The percent inhibition of schizont maturation was estimated as the number of unreleased schizonts versus the number in control parasite cultures without inhibitor treatment. The experiments were repeated three times, and the final results were averages from the three experiments.

FIG 2  (A) SDS-PAGE analysis of the purified recombinant N-terminal domain of PIMRS\textsuperscript{a} (residues 1 to 232, corresponding to a 28-kDa band) (left) and expression of PIMRS\textsuperscript{a} in parasite lysates assessed using antibodies against this N-terminal domain (right). (B) Purified maltose binding protein (MBP)-tagged anticodon binding domain (residues 584 to 704) of PIMRS\textsuperscript{a}. Left, SDS-PAGE gel, showing bands at 57 kDa, corresponding to the MBP-fused PIMRS\textsuperscript{a} anticodon binding domain, and at 43 kDa, corresponding to MBP. Right, Western blot of PIMRS\textsuperscript{a} in parasite lysates using antibodies against the anticodon binding domain. Lanes 1 in panels A and B show protein molecular weight markers, and lanes 2 show purified recombinant protein. (C) Localization of PIMRS\textsuperscript{a} using antibodies directed against the N-terminal domain. The protein localizes in the cytoplasm for all asexual parasite stages. (D) Localization of MRS\textsuperscript{a}, studied using antibodies against the anticodon binding domain. Localization of MRS\textsuperscript{a} in apicoplasts in three asexual stages (ring, trophozoite, and schizont) was verified.
In vitro transcription of tRNA\textsuperscript{Met} and preparation of total protein extracts from parasites. The sequence for tRNA\textsuperscript{Met} was taken from the PlasmoDB database and was synthesized commercially with the addition of a T7 promoter site in the 5\textsuperscript{′} region. The tRNA was prepared from a double-stranded DNA template. The last two bases in the reverse primer were modified with 2-O\textsuperscript{−}methyl substitution at the hydroxyl group of the ribose sugar. The tRNA was then synthesized using an in vitro transcription kit (New England BioLabs), by incubating the reaction mixture for 10 h at 37°C. The tRNA was isolated by ethanol precipitation in the presence of 0.3 M sodium acetate and was resuspended in RNase-free water.

To extract total protein from \textit{Plasmodium falciparum} 3D7 parasites, RBCs were lysed with 0.15% saponin and centrifuged at 14,000 rpm for 10 min at 4°C to separate the parasites. These cells were then lysed on ice for 10 min with 0.65% NP-40 (HiMedia) in buffer A, containing 30 mM HEPES (pH 7.5), 140 mM NaCl, 30 mM KCl, 40 mM MgCl\textsubscript{2}, and 1 mM dithiothreitol (DTT), supplemented with protease inhibitors. Protein extracts were stored at −80°C.

\textbf{RESULTS}

\textbf{Domain organization of PfMRS\textsuperscript{cyt} and PfMRS\textsuperscript{api} and comparison with other MRSs.} The \textit{Plasmodium} species possess two copies of PfMRS\textsuperscript{cyt} and PfMRS\textsuperscript{api}, \textit{P. falciparum} 3D7 cells was assayed for activity in the presence of tRNA\textsuperscript{Met} prepared \textit{in vitro}, using the malachite green assay described by Cestari and Stuart (39), with some modifications. Fold increases in \(P_i\) release were assessed by performing the aminocacylation reaction in a time-dependent manner in the absence and presence of tRNA\textsuperscript{Met}.

To determine the effects of inhibitors on aminocacylation, reactions were performed in 50-μl volumes in 96-well flat-bottom plates containing 100 μg total protein extract. The inhibitors were added at serially diluted concentrations (0.1 nM to 100 μM) and incubated with protein extract for 15 min at 37°C in buffer A. The reaction mixture was then supplemented with 1 μM tRNA\textsuperscript{Met} 100 μM ATP, 50 μM methionine, and 2 U/ml pyrophosphatase (PPase) in buffer A and incubated for 10 min at 37°C. The reaction was stopped with the addition of one-fourth of the total reaction volume of malachite green reagent. Reactions performed in the absence of inhibitors were used as controls. Pyrophosphate release was quantified by the formation of complexes between malachite green molybdate and free orthophosphate. The absorbance was read at 620 nm using a 96-well enzyme-linked immunosorbent assay (ELISA) plate reader (Versamax; Molecular Biosystems). Absorbance values were compared with a standard curve, and results were plotted as percent decrease in PfMRS\textsuperscript{cyt} activity versus inhibitor concentration.
of MRS enzymes encoded within their nuclear genomes. In the case of P. falciparum, the genome contains the genes PF10_0340 (PfMRScyt) and PF10_0053 (PfMRSapi) in PlasmoDB, with each PfMRS having a distinct domain architecture (Fig. 1). Both proteins expectedly show conserved motifs such as HIGH and KMSKS, which are characteristic of class I aaRSs. However, the sequences of the synthetase subdomains of MRScyt and MRSapi are only ~26% identical to each other. In addition to the synthetase domain of 497 residues in MRScyt, domains of 225 residues at the N terminus and 167 residues at the C terminus are appended to the core catalytic domain. In yeast and humans, the structural folding of the N-terminal MRScyt domain is similar to nonenzymatic glutathione S-transferase (GST) folding. The presence of the GST domain may be meant to mediate interactions of MRS with other proteins containing GST domains (40). Although the GST domain in PfMRScyt is conserved among Plasmodium species, it does not show any significant similarity to orthologous MRS proteins. On the other hand, MRSapi has a unique low-complexity insertion region of 35 amino acids in the connective peptide 1 (CP1) region and a highly divergent sequence of 140 residues at the N terminus (of 609 residues). Furthermore, and rather surprisingly, the N-terminal extension in MRSapi is absent in Plasmodium vivax, suggesting unusual intraspecies variation in this set of housekeeping enzymes (Fig. 1).

Localization of MRScyt and MRSapi in malaria parasites. Despite extensive efforts, recombinant production of both PfMRSs was not successful in our laboratory. Therefore, for localization studies, polyclonal antibodies were raised against the recombinantly expressed N- and C-terminal domains of PfMRScyt and tRNA binding domain of PfMRSapi. These antibodies were used to study, using confocal microscopy, the localization of the respective proteins in all asexual stages of malaria parasites. The expression of both PfMRSs in parasites was confirmed using these antibodies (Fig. 2A and B; also see Fig. S1B in the supplemental material). The microscopy data revealed the presence of PfMRScyt solely in the cytoplasm of asexual stages, including merozoites (Fig. 2C; also see Fig. S1C, D, and F in the supplemental material). In contrast to human methionyl-tRNA synthetase, which shows nuclear localization in the presence of growth factors (41), translocation of PfMRScyt was not observed in any of the asexual stages even in the presence of growth factors (data not shown). In all asexual blood stages of parasite development, PfMRSapi showed distinct localization in the apicoplasts of the parasites (Fig. 2D; also see Fig. S1E in the supplemental material). From these localization studies, it is evident that PfMRSs are compartmentalized within the parasite cytoplasm and apicoplasts, to assist in protein translation in these compartments.

Evolutionary relationships of PfMRScyt and PfMRSapi. The differential localization of PfMRSs prompted us to analyze the phylogenetic relationships of MRSs in various taxa. This analysis covered organisms from primitive bacteria, fungi, and algae to complex eukaryotes. To avoid any bias in the data, we trimmed the N- and C-terminal-appended domains, as they do not share any homology with sequences of known MRSs. Similarly, a low-complexity region (LCR) of 35 amino acids in PfMRSapi was removed. The phylogenetic data revealed a lower-plant-like origin of
PfMRScyt, whereas PfMRSapi showed close similarity to fungus-like organisms. The synthetase domains of the two PfMRSs showed a polyphyletic relationship with each other. The apicomplexan MRScyt included in our analysis fell close to heterokonts such as *Phytopthora* and *Thalassiosira*, while MRSapi appeared in a fungal clade of the phylogenetic tree. PfMRSapi and *Leishmania* major MRS are also closely related to primitive thermophilic and nitrogen-fixing bacteria, sharing a common ancestor (see Fig. S2 in the supplemental material). It was unexpected to find fungal and primitive bacterial associations of MRSapi, as this enzyme resides in parasite apicoplasts and was expected to be of plant-like origin, owing to the plastid-like nature of apicoplasts. The distinct evolutionary history of MRScyt and MRSapi suggests that the two proteins have not originated as a result of a gene duplication event and are not paralogs.

Modeling of PfMRS synthetase domains and *in silico* inhibitor screening against PfMRScyt. In an attempt to target parasite MRSapi, we modeled the synthetase domains of both PfMRSs. The levels of sequence identity with *Thermos thermophilus* MRS (PDB accession number 1A8H) were 37% for PfMRScyt and 28% for PfMRSapi. Therefore, *T. thermophilus* MRS was used as the template (see Fig. S3 in the supplemental material). A structure-based sequence alignment revealed several differences in the active site residues involved in binding the l-methionyl adenylate (MOD) complex. Furthermore, some of the MOD binding residues in PfMRScyt were dissimilar with respect to the human cytoplasmic counterpart. Therefore, we decided to screen the PfMRScyt active site for small-molecule compounds that could serve as inhibitors of this enzyme. We screened the whole active site pocket, including the MOD binding pocket and the auxiliary pocket in the l-Met binding site. The presence of an auxiliary pocket and an extended l-Met binding pocket was reported previously for *Trypanosoma brucei* MRS (42).

A small-molecule library (~50,000 compounds; Specs) was screened and top-ranking ligands were selected based on docking scores and bonding interactions. A total of 40 compounds were procured for *in vitro* parasite growth inhibition assays, and several compounds showed submicromolar parasite growth inhibition (see Fig. 5; also see Table S1 in the supplemental material). Analogs of these compounds were purchased and further screened in parasite growth inhibition assays.

**Parasite growth inhibition by REP3123 and REP8839.** REP3123 and REP8839 are known inhibitors of bacterial MRSs and show potent and selective activity, with minimal cytotoxicity for mammalian cells (43). REP3123 is active against various Gram-positive and Gram-negative bacteria, including resistant *Clostridium* stains and *Staphylococcus* strains. REP3123 kills *Clostridium difficile* with a Kᵢ of 0.02 nM, compared with values of 0.017 nM and 0.08 nM against *Staphylococcus aureus* and *Staphylococcus pneumoniae*, respectively, *in vitro*. REP8839 inhibits MRS...
activity with a $K_i$ of 10 pM against Staphylococcus aureus \cite{23, 24}. Therefore, we tested the effects of these MRS inhibitors on malaria parasite growth in vitro. The experiments were performed with the Plasmodium falciparum 3D7 strain at ~1% parasitemia and 2% hematocrit levels. Both of the compounds were found to inhibit parasite growth and developmental progression. REP3123 inhibited parasite growth with an EC$_{50}$ of 144 nM, while REP8839 showed the same effect at 155 nM (Fig. 3A and B). The two compounds also seemed to affect parasite maturation in early and late developmental stages. The compounds inhibited parasite development in the early growth stages at approximately the IC$_{70}$ (500 nM) and prevented the formation of mature trophozoites and schizonts (Fig. 3C). Both compounds were also found to inhibit maturation in the late stages of parasite development. The inhibitors were used to treat late trophozoites or early schizonts (~40 h post-invasion). Synchronized iRBCs at 0.5 to 1% parasitemia were incubated with 200 nM or 500 nM inhibitors for 24 h. REP3123 and REP8839 prevented schizont maturation and subsequent rupture by ~65% and 58%, respectively, at 200 nM. The inhibition of maturation was improved at approximately the IC$_{70}$ (500 nM), at which both MRS inhibitors reduced parasite development by ~75% (Fig. 4). Interestingly, both of these MRS inhibitors are in clinical trials for the treatment of bacterial infections \cite{24}, and their derivatives may be valuable starting points for targeting of PfMRSs.

**Parasite growth inhibition using compounds screened in silico.** The compounds were dissolved in DMSO (not exceeding a final concentration of 1% in parasite cultures) and tested for activity against parasite cultures with the 3D7 strain. The experiments were performed in a 96-well format with a concentration range of 0.1 nM to 100 µM, in triplicate. Estimations of parasite growth inhibition were performed with SYBR green assays. Several compounds were found to affect parasite multiplication in vitro (Fig. 5; also see Table S1 in the supplemental material), and a few of those compounds showed EC$_{50}$s in the nanomolar or sub-micromolar range. The compounds C1, C2, and C3 and their analogs showed EC$_{50}$ values of ~500 nM (Fig. 6A and D). The other compounds listed in Fig. 5 with inhibitory effects on parasite growth are analogs of these three compounds. The effects of these inhibitors were observed by labeling parasite DNA with SYBR green, after 48 h of incubation. All compounds killed parasites in the first cycle of erythrocytic development, suggesting that their likely target was PfMRS$^{35}$ and not the apicoplastic PfMRS. We also tested the effects of the three selected inhibitors on parasite growth progression at IC$_{70}$ levels in a 48-h cycle. The three compounds were found to effectively inhibit parasite progression from the ring stage to the trophozoite stage, and this effect was observed in nearly 70 to 80% of iRBCs (Fig. 6B).
Protein labeling with $^{35}$S-labeled methionine and cysteine. To investigate whether the selected compounds C1, C2, and C3 were specific for PfMRS and would thereby abrogate protein synthesis, we added $^{35}$S-labeled methionine and cysteine to the culture medium, to label newly synthesized proteins. The incorporation of these labeled amino acids was analyzed after starving of the cells for 30 min in methionine- and cysteine-free RPMI 1640 medium, in the presence of inhibitors at concentrations of 0.1 nM to 100 μM. We used anisomycin, a widely used inhibitor of protein synthesis in eukaryotes (44), as a positive control and pyrimethamine, a known parasite growth inhibitor that specifically targets folate acid metabolism (45), as a negative control. Cells without inhibitors or drugs were considered to demonstrate 100% incorporation of $^{35}$S-labeled amino acids. Treatment with the selected compounds quickly decreased protein synthesis in the parasites. The reductions in protein synthesis were ~50% in 1 h with EC$_{50}$ levels of the three inhibitors (Fig. 6C). A comparison of EC$_{50}$ against parasite growth and deficits in protein synthesis with C1, C2, and C3 is presented in Table 1. These experiments suggest the likelihood of the selected compounds specifically targeting the protein translational machinery via inhibition of PfMRS.

PfMRS enzyme activity assays with inhibitors. Parasites were lysed for 10 min with 0.65% NP-40 on ice and were centrifuged to separate the cytosolic fraction from other membrane-bound organelles (46). Inhibition of PfMRS aminoacylation activity was performed in the presence of identified inhibitors, using protein extracts as the source of PfMRS. DNA strands (see Fig. S4A in the supplemental material) encoding tRNA$^{\text{Met}}$ (PlasmoDB accession number PF3D7_1339100) were synthesized for this experiment. The last two bases in the reverse primer were modified by incorporation of a methyl group at the 2'-OH group of the ribose sugar, and tRNA$^{\text{Met}}$ was prepared using an in vitro transcription kit, as given in the reaction scheme (see Fig. S4B in the supplemental material). The malachite green assay (which quantifies the release of P$_{i}$ as a result of the aminoacylation reaction) was performed in the presence or absence of tRNA$^{\text{Met}}$. A 4-fold increase in activity was observed with the addition of external tRNA (see Fig. S4C in the supplemental material). Inhibitors were incubated with parasite protein extracts for 15 min at 37°C in buffer A (30 mM HEPES [pH 7.5], 140 mM NaCl, 30 mM KCl, 40 mM MgCl$_2$, and 1 mM DTT). The reaction mixture was then supplemented with 50 μM methionine, 100 μM ATP, and 1 μM tRNA$^{\text{Met}}$ for 10 min at 37°C. The quantified P$_{i}$ release was plotted as a percentage with respect to values for the reaction performed in the absence of inhibitors. Significant decreases in activity were observed with increasing concentrations of inhibitors. REP3123 and REP8839 blocked PfMRS activity with EC$_{50}$ values of ~146 and 281 nM, respectively (Fig. 7A). Nonlinear regression with the variable slope method, following the equation $y = 100/(1 + 10^{(\log IC_{50} - x) \times \text{Hill slope}})$ (in which IC$_{50}$ is the 50% inhibitory concentration), was used to fit the curves. All graphs were plotted using GraphPad Prism 6 software.

The crystal structure of REP3123 in complex with Clostridium difficile MRS (47) reveals the molecular basis of enzyme inhibition, as this drug binds in the methionine binding pocket and into an adjacent nonsubstrate binding pocket. Of the hit compounds we studied, C2 inhibited the enzyme activity with an EC$_{50}$ of 154 nM (Fig. 7B), while C1 and C3 inhibited the enzyme activity with values of ~571 and 614 nM, respectively, and are predicted to bind to the MRS active site in a manner similar to that of REP3123. The activity assays with parasite lysates showed no inhibition if the assays were carried out in the presence of 1 mM methionine, indicating possible competition with the methionine binding site (data not shown).

Binding modes of hit compounds in the MRS active site. The three effective compounds (C1, C2, and C3) were reaxed into the active site of MRS$^{\text{35}}$ using Glide XP in the Schrödinger suite, to study their binding modes. Visual analysis suggested that these compounds are stabilized in the active site pocket by extensive hydrophobic interactions and they show distinct modes of binding. The binding pocket for C1 is located between the extended l-Met binding site and the auxiliary pocket instead of the ATP binding site (Fig. 8A). The naphthalene ring of compound C1 is stabilized by Phe482, His483, Tyr454, Ile231, Leu451, and Trp447, while its piperidine is stabilized by hydrogen bonding interactions with Asp270 (Fig. 8B). The binding mode of compound C2 is similar to methionyl adenylate (MOD) binding, and its benzimidazole ring mimics the
adenine moiety of MOD, which is stabilized by hydrophobic interactions with His505, His42, Leu509, and Phe507 (Fig. 8C). The middle aromatic ring in compound C2 is stabilized by hydrophobic interactions with His483, Ile479, and Ile231. The halogen-substituted ring occupies a hydrophobic pocket formed by extended L-Met binding pocket residues Tyr454, Phe482, Trp447, Leu451, and Val446. The halogen (Cl and F) atoms in compound C2 are stabilized by hydrogen bonding interactions with Glu351 and Asp270, respectively (Fig. 8D).

The halogen-substituted aromatic ring of compound C3 occupies an extended L-Met binding pocket similar to that of the halogen-substituted ring in compound C2 (Fig. 8E). The amino group-containing chain is stabilized by bifurcated hydrogen bonding interactions with Asp270 and Glu351 (Fig. 8F).

**DISCUSSION**

To fulfill the requirements of protein synthesis, malaria parasite protein translation enzymes and their reaction substrates and products need to be distributed between apicoplasts, mitochondria, and cytoplasm (7, 8, 13, 14, 22). In *P. falciparum*, dual localization of single-copy aaRSs has been observed in the cases of Ala-RS, Gly-RS, Thr-RS, and Cys-RS (7, 8, 13, 14, 23). However, several *P. falciparum* aaRSs remain unannotated experimentally, in terms of their cellular localization. PfMRSs typify this problem, as the presence of two copies in the parasite genome alerted us to the possibility of dual compartmentalization (7, 8). Our results demonstrate that one copy, PfMRS\textsuperscript{api}, is targeted to the parasite apicoplasts, while the other, PfMRS\textsuperscript{cyt}, resides in the parasite cytoplasm in asexual blood-stage parasites. The homolog of...
From our phylogenetic tree, it is apparent that PfMRScyt and cytoplasm and apicoplasts, presumably serving as initiator and these tRNAs are predicted to be compartmentalized to parasite dinium has four copies of tRNAs specific for MRSs. Two each of

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