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Selective Inhibition of an Apicoplastic Aminoacyl-tRNA Synthetase from *Plasmodium falciparum*

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The resistance of malaria parasites to available drugs continues to grow, and this makes the need for new antimalarial therapies pressing. Aminoacyl-tRNA synthetases (ARSs) are essential enzymes and well-established antibacterial targets and so constitute a promising set of targets for the development of new antimalarials. Despite their potential as drug targets, apicoplastic ARSs remain unexplored. We have characterized the lysylation system of *Plasmodium falciparum*, and designed, synthesized, and tested a set of inhibitors based on the structure of the natural substrate intermediate: lysyl-adenylate. Here we demonstrate that selective inhibition of apicoplastic ARSs is feasible and describe new compounds that that specifically inhibit *Plasmodium* apicoplastic lysyl-tRNA synthetase and show antimalarial activities in the micromolar range.

Introduction

Malaria remains one of the most important infectious diseases in the world, causing acute illness in more than 100 million people and leading to approximately 1 million deaths annually.[1] In addition to its human cost, malaria represents a massive economic burden, contributing substantially to poverty in the developing world. Effective antimalarial drugs are available, but their efficacy is compromised by emerging resistance.[2] There is therefore a broad consensus about the need to develop new antimalarial drugs. Malaria is caused by *Plasmodium*, a genus of parasitic protists. At the moment there are over 200 species known of this genus, of which at least 11 can infect humans. Of these, *Plasmodium falciparum* causes the most severe form of malaria, being responsible for 90% of the deaths.[1]

The *P. falciparum* genome project revealed many new potential drug targets,[3–9] several of which are enzymes that act in the apicoplast, a relict plastid derived from secondary endosymbiosis of cyanobacteria,[10] which is essential for the parasite's survival.[11,12] Many of its bacterial-like enzymes are substantially different from their mammalian homologues,[13–15] and this makes them excellent drug target candidates. Several antibacterial drugs that are clinically used for the treatment of malaria and toxoplasmosis (e.g., doxycycline, clindamycin, and spiramycin) act on apicoplastic targets. These drugs typically display a "delayed death" phenotype, which is characterized by the inhibition of parasite growth on the second erythrocytic cycle after the drug treatment.[16–20]

Aminoacyl-tRNA synthetases (ARSs) are essential enzymes and well-established antimicrobial drug targets,[21,22] and so represent interesting new targets for antimalarial drug discovery.[23] They perform a central role in the translation of the genetic code by catalyzing the attachment of each amino acid to its cognate transfer RNA (tRNA). Although these enzymes differ widely in size, sequence, and oligomeric state, they all carry out similar two-step reactions.[22] In a first step, the ARS catalyzes the activation of the amino acid, and in a second step the aminoacyl-adenylate intermediate (AA-AMP) is transferred to the tRNA.

1) $AA + ATP \rightarrow AA\text{-}AMP + PP_i$
2) $AA\text{-}AMP + tRNA \rightarrow AA\text{-}tRNA + AMP$

Currently, ARS inhibition is the mechanism of action of one commercially available antibiotic: pseudomonic acid or mupirocin (GlaxoSmithKline), a natural product that inhibits bacterial isoleucyl-tRNA synthetases with an 8000-fold selectivity over

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their mammalian homologues. Mupirocin has also been shown to inhibit *Plasmodium* apicoplastic isoleucyl-tRNA synthetase.[24] Other ARS inhibitors described to date include natural products, such as borrelidin,[25,26] granaticin,[27] indolmycin,[28] furanomycin,[29] ochratoxin A,[30] and cispentacin,[31] as well as several semisynthetic products.[32–34] Most efforts in the design of new synthetic drugs targeting ARSs have focused on mimicking the aminoacyl adenylate intermediates (AA-AMPs).[21,35–37] Finally, it has recently been reported that cladosporin, a fungal secondary metabolite, targets *P. falciparum* cytosolic lysyl-tRNA synthetase (PfKRS-1) with a selectivity of 100-fold with respect to its human homologue.[38]

Indeed, the main challenge in using ARSs as drug targets is to avoid cross-reactivity with their human homologues. In this regard, apicoplast-specific *P. falciparum* lysyl-tRNA synthetase (PfKRS-2) is interesting because its cyanobacterial origin makes it evolutionarily distant from human lysyl-tRNA synthetase (HsKRS). In this work we present a new series of compounds that selectively inhibit apicoplastic PfKRS-2, thus validating its potential as an antimalarial drug target, and demonstrating that specific inhibition of apicoplastic ARS is feasible.

Results

Characterization of the lysylation system in *P. falciparum*

Malaria parasites possess two distinct lysyl-tRNA synthetases: PfKRS-1 (PF13_0262), and PfKRS-2 (PF14_0166). Subcellular localization prediction software[11] finds that PfKRS-1 would be expected to be cytosolic, whereas PfKRS-2 would be expected to be targeted to the apicoplast (Figure 1 A). Immunofluorescence assays on PfKRS-2_{leader}-GFP-transfected *P. falciparum* parasites (Figure 1 B) indicate that, as expected, PfKRS-2 is exclusively located in the apicoplast.

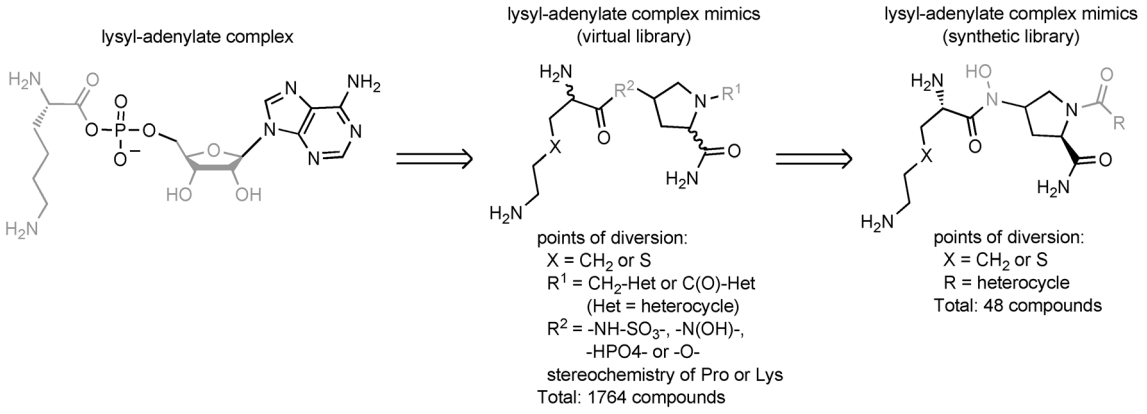
In general, apicoplastic-targeted enzymes tend to be of bacterial origin.[39] To confirm the bacterial origin of PfKRS-2 and to evaluate its evolutionary distance from its human homologue, we performed a structure-based phylogenetic analysis on class II lysyl-tRNA synthetases from all kingdoms (Figure 1 C). Our results show that apicoplastic lysyl-tRNA synthetases cluster with bacterial enzymes and are only distantly related to HsKRS.

Using a manually curated homology model of PfKRS-2 (Figure S1 in the Supporting Information), we noted that those residues involved in the recognition of lysine in the bacterial, human, and *P. falciparum* enzymes are conserved across species. Importantly, however, other residues in the active site cavity that are not involved in substrate recognition are not so well conserved, and the sizes of the catalytic cavities are significantly different (Figure 1 D). This suggests that the active site in PfKRS-2 might be able to accommodate ligands that might not be able to bind in the HsKRS cavity, due to steric restrictions. Altogether, our analyses suggest that specific design of inhibitors to target the active site of PfKRS-2 is feasible.

Design, selection, and synthesis of a library of lysyl-adenylate analogues

A virtual compound library was designed in order to identify molecules that might mimic the lysyl-adenylate intermediate (Scheme 1). To construct the library, a proline derivative was used as a ribose mimetic, together with a heterocycle as an adenylate substitute, as previously described.[36] In addition, four more points of chemical diversity were explored: 1) both lysine and thialysine derivatives were used as lysine analogues, 2) the phosphate linker was replaced by other types of chemical linkers, 3) heterocyclic groups were used as substitutes for the adenylate moiety, and 4) the stereochemistry of the proline and the lysine derivatives was varied. With this approach a library of 1764 compounds was designed and evaluated by docking the molecules against the 3D structures of both PfKRS-2 and HsKRS.

All 1764 compounds were docked both to PfKRS-2 and HsKRS, and the different ligand poses obtained were ranked by use of GlideScore.[40] Compounds to be synthesized for experimental testing were selected on the basis of their selectivity towards the PfKRS-2 enzyme. By this criterion we selected 36 compounds for further analysis (Table S1).



Scheme 1. Design of the virtual library and the synthetic library. The reaction intermediate lysyl-adenylate (left) was subdivided into four parts, which have been shaded accordingly. A virtual library of 1764 compounds (middle) based on the structure of the lysyl-adenylate complex (generic structure in (*R*,*R*) configuration is depicted) was constructed. On the basis of the docking predictions, a library of 48 compounds was built (right).

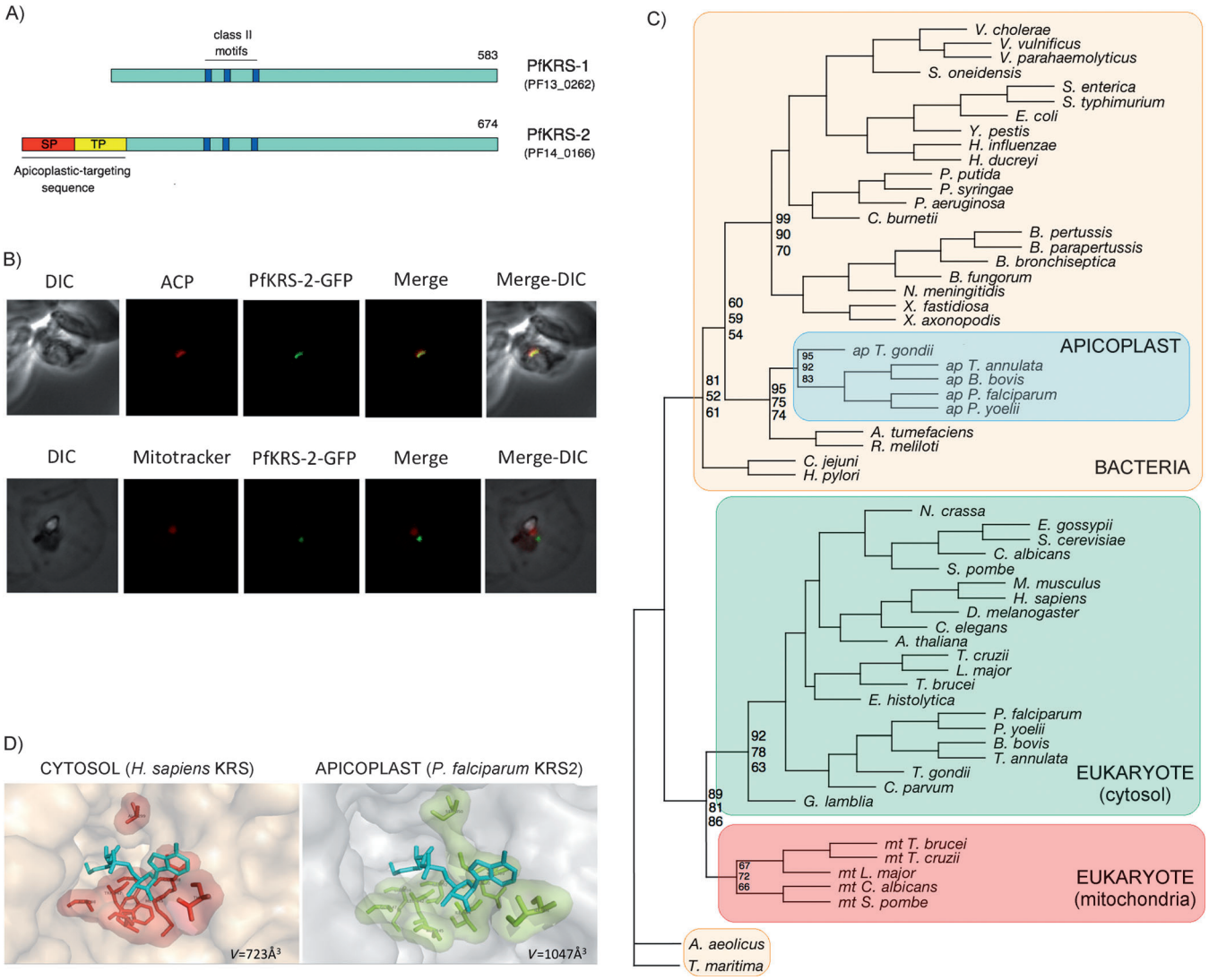


Figure 1. Characterization of the lysylation system in *P. falciparum*. A) Domain structures of *P. falciparum* KRS and KRS-2. The bipartite apicoplastic-targeting signal, consisting of signal peptide (SP) followed by a transit peptide (TP), is only found in PF14_0166; this suggests that PF13_0262 corresponds to the cytosolic enzyme, whereas PF14_0166 corresponds to the apicoplastic enzyme. B) Immunofluorescence assays of infected red blood cells (iRBCs) containing PfKRS-2_{leader}-GFP-transfected *P. falciparum* parasites. The GFP-tagged sequence colocalizes with ACP and not with Mitotracker, indicating that it is being specifically targeted to the apicoplast, consistently with the bioinformatic predictions. C) Phylogenetic analysis of class II lysyl-tRNA synthetases. The plasmodial PfKRS-1 and PfKRS-2 are boxed in red, whereas the human HsKRS is boxed in blue. PfKRS-2 clusters with bacterial sequences, whereas PfKRS-1 and HsKRS cluster with eukaryotic sequences. D) Active site comparison between PfKRS2 and its human homologue. The active site is defined as those residues with at least one of their atoms at less than 4 Å from the ligand. Differing residues are highlighted. See also Figure S1.

Amongst the 36 compounds selected, 70% contained a hydroxamate group as a phosphate analogue and a proline ring with an (*S,S*) configuration. A library of 50 lysyl-adenylate analogues based on the (*S,S*)-4-aminoproline scaffold with a hydroxamate group as a phosphate linker mimic was thus designed. Of these compounds, 25 contained lysine, whereas the other 25 contained thialysine (Table 1; see also Scheme 1). In addition to the predicted hits, a number of predicted nonselective and inactive compounds were also synthesized to evaluate the performance of the docking calculations (Figure S2 B).

The library of potential PfKRS-2 inhibitors was produced by solid-phase synthesis based on an Alloc/Boc strategy (Scheme 2).[36] Coupling of Alloc-protected hydroxyproline to the resin was followed by the introduction of the protected lysine or thialysine hydroxamic acid moiety under Mitsunobu conditions. After removal of the Alloc group with $Pd(PPh_3)_4$ and $PhSiH_3$, introduction of the different carboxylic acids was carried out under standard peptide coupling conditions. Subsequent cleavage of the products and protecting group removal under strongly acidic condition produced the crude inhibitors. Purification by preparative HPLC yielded the desired products with purities of $\geq$85% (Scheme 2 A). A number of thialysine-derived products were not obtained, due to degradation of the products during purification. The obtained products were subjected to biological evaluation.

Table 1. List of 46 synthesized compounds, ranked by their GlideScores when docked into the PfKRS-2 homology model (Pf-Gscore). The GlideScores in the case of the human homologue (Hs-Gscore) are also shown. See also Figure S2 and Table S1.

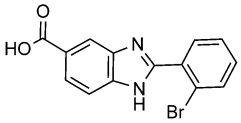
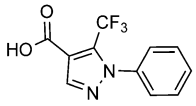
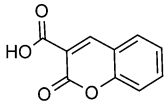
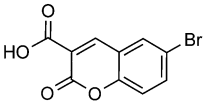
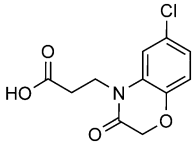
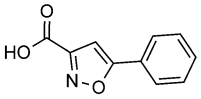
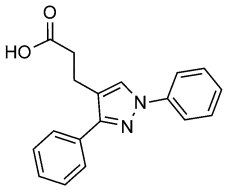
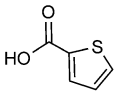
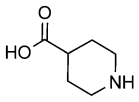
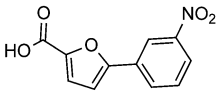
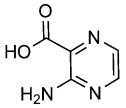
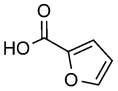
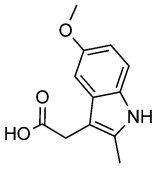
	Lysine derivatives					Thialysine derivatives				
R group	Code[a]	Pf-Gscore	Hs-Gscore	Yield [mg]	Purity [%]	Code	Pf-Gscore	Hs-Gscore	Yield [mg]	Purity [%]
	M-01	−12.94	−9.63	3.5	84.4	**M-26**	−11.99	−10.85	3.2	96.0
	M-02	−12.23	−8.85	9.3	95.0	M-27	−11.06	−7.94	8.1	90.9
	M-03	−10.76	−10.32	1.9	53.0	M-28	−9.28	−8.40	–	–
	M-04	−11.46	−9.66	1.6	90.2	M-29	−10.88	−10.60	–	–
	M-06	−11.22	−9.91	3.9	98.7	M-31	−9.68	−9.73	–	–
	M-07	−11.74	−9.40	4.0	93.9	M-32	−10.43	−8.40	11.2	98.5
	M-08	−12.70	−8.86	1.1	90.2	**M-33**	−12.00	−9.89	6.5	92.0
	M-09	−9.38	−8.63	3.4	91.4	M-34	−9.25	−10.89	5.1	>99.9
	M-11	−10.50	−8.68	12.0	85.8	M-36	−9.75	−9.50	10.1	96.9
	**M-12**	−10.97	−9.12	6.3	89.4	**M-37**	−10.54	−8.23	8.3	88.2
	M-13	−11.00	−8.88	10.4	92.7	M-38	−10.54	−8.23	20.1	86.8
	M-14	−11.76	−8.23	4.2	86.5	M-39	−9.84	−9.37	12.6	84.9
	M-15	−9.78	−10.68	1.4	99.0	M-40	−10.42	−10.66	12.7	90.0

Table 1. (Continued)

R group	Lysine derivatives					Thialysine derivatives				
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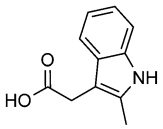
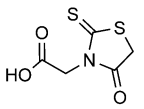
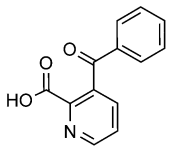
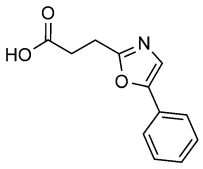
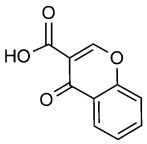
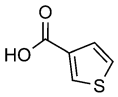
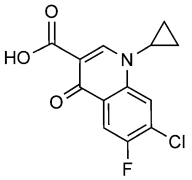
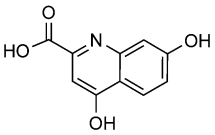
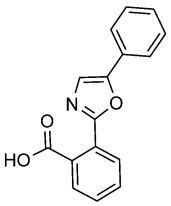
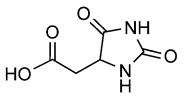
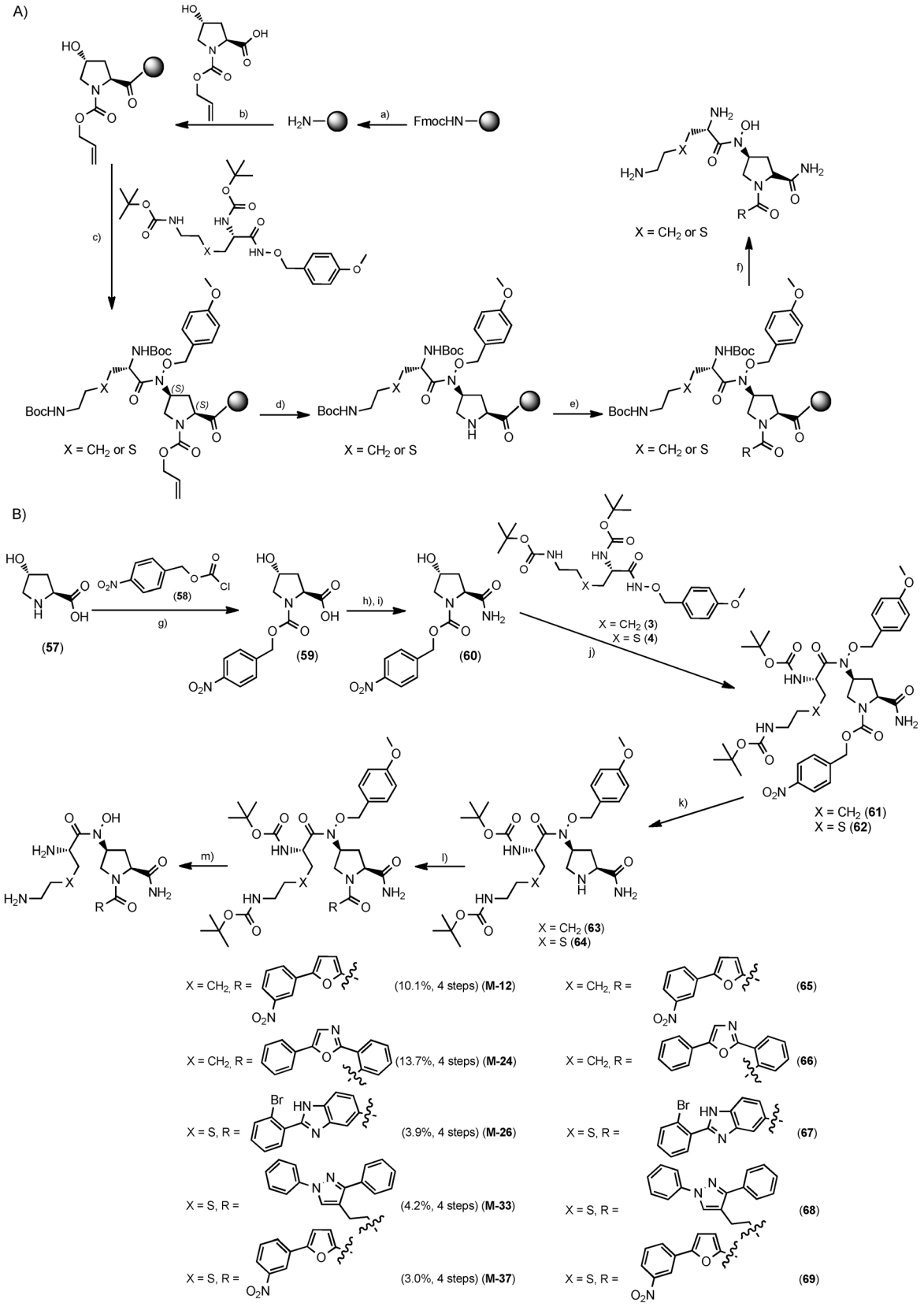
	M-16	−10.43	−11.62	0.9	93.9	M-41	−10.11	−8.38	11.4	88.1
	M-17	−9.89	−9.42	1.0	95.3	M-42	−9.89	−10.21	4.9	92.8
	M-18	−9.59	−10.55	4.2	86.7	M-43	−9.66	−9.83	8.6	92.6
	M-19	−10.26	−8.12	2.0	86.5	M-44	−11.17	−9.54	3.9	96.5
	M-20	−10.22	−8.86	1.3	95.3	M-45	−10.49	−9.10	–	–
	M-21	−9.75	−11.09	1.7	76.5	M-46	−9.99	−8.54	6.5	91.3
	M-22	−10.62	−10.69	2.0	89.5	M-47	−12.49	−9.85	9.0	92.7
	M-23	−11.19	−10.45	3.0	95.9	M-48	−9.94	−11.59	3.5	93.1
	**M-24**	−9.52	−11.16	0.5	99.0	M-49	−9.79	−10.53	9.0	95.8
	M-25	−9.59	−10.77	4.7	92.0	M-50	−10.08	−8.90	–	–

[a] The subset of selected compounds that were resynthesized for further in vitro analyses is shown in bold.



Scheme 2. Synthesis of the library of lysyl-adenylate analogues. A) Solid-phase synthesis of the library. a) 20% piperidine in DMF; b) HOBt, DiPCDI, DMF; c) DIAD, CH_2Cl_2, PPh_3; d) $Pd(PPh_3)_4$, $PhSiH_3$, CH_2Cl_2, 2×20′; e) RCOOH, HOBt, DIPCDI, DMF, 2 h; f) TFA/CH_2Cl_2/TIS (95:2.5:2.5). B) Synthesis of the active compounds in solution. g) 2 M NaOH(aq), CH_2Cl_2 (57%); h) WSC·HCl and HOBt·H_2O; i) 32% NH_4OH(aq) (66%); j) PPh_3, DEAD, THF; k) 10% Pd/C, H_2, MeOH; l) WSC·HCl, HOBt·H_2O, DMF/DMF (9:1), RCOOH; m) 40% TFA in CH_2Cl_2. See also Figure S4.

Table 2. In vitro inhibition of the five resynthesized compounds.[a] See also Figure S4.

	Screening (purity > 85%, 150 μM)					Resynthesized hits (purity > 95%, 50 μM)					
	Purity	LDH [% inhib.]		Smears [% inhib.]		Purity	Smears [% inhib.]		IC_{50} [μM]		Selectivity
	[%]	48 h	96 h	48 h	96 h	[%]	48 h	96 h	48 h	96 h	[fold]
M-12	93	0	45.9	23.2	58.0	99.5	31.7	70.4	172	83.2	2.2
M-24	92	4.42	58.7	0	58.3	99.5	32.6	42.4	518	427.1	1.2
M-26	96	0	35.5	0.18	66.0	100	3.90	41.6	551	84.7	6.5
M-33	85	24.7	100	76.6	98.9	98.6	43.5	77.9	48.1	29.5	1.4
M-37	88	15.7	65.5	49.7	100	100	6.20	72.7	151	38.4	3.9

[a] Inhibition of *P. falciparum* cultures measured by: 1) LDH (lactate dehydrogenase) assay, and 2) visual inspection of Giemsa-stained smears.

In vitro testing of the compounds

All of the synthesized compounds were initially tested by the pLDH assay for their ability to kill *P. falciparum* parasites.[41] Inhibitors of apicoplastic protein synthesis kill the parasite in a retarded manner,[16–18] so we used the "delayed death" phenotype as an initial indication that a compound in our library might be preferentially targeting apicoplastic lysyl-tRNA synthetase. Our initial screening allowed us to select five compounds that showed clear delayed inhibitory effects (Table 2). The activities of these compounds were further confirmed by visual inspection of smears.

The five most active compounds from the library (**M-12**, **M-24**, **M-26**, **M-33**, and **M-37**) were resynthesized. Solution synthesis was used to improve purities and yields and to minimize possible side-reactions (Scheme 2B). All products were obtained in purities of > 98.5%. The antimalarial activities of the resynthesized compounds were evaluated by visual analysis of *P. falciparum* smears. The highest inhibition rates were observed for compounds **M-12**, **M-33**, and **M-37** (Table 2). In order to select specific inhibitors of the apicoplastic translation machinery it was decided to focus on those compounds that produced clear delayed effect phenomena. Compounds **M-26** and **M-37** were thus chosen as drug candidates for further in vitro and in vivo analyses, given that these compounds show the greatest differences in their inhibitory rates at 48 and 96 h.

In order to investigate the selectivities and specificities of **M-26** and **M-37**, we first verified their abilities to inhibit HsKRS, PfKRS-1, and PfKRS-2. In vitro aminoacylation assays were performed with radiolabeled lysine and in-vitro-transcribed $tRNA^{Lys}$, and the effects of the compounds upon these aminoacylation reactions were quantified. Both **M-26** and **M-37** were found to inhibit PfKRS-2, but were not active against HsKRS or PfKRS-1 (Figure 2), in accordance with our docking predictions. We also verified that the compounds did not inhibit other plasmodial ARSs (Figure S2). We can therefore conclude that **M-26** and **M-37** are selective inhibitors of apicoplastic PfKRS-2.

Structural basis for selectivity

Through analysis of the binding mode of the natural lysyl-adenylate (LAD) ligand, it was observed that both the adenine and the lysine moieties of LAD are recognized at the binding site and are major contributors to the free energy of binding of the reaction intermediate. In agreement with this observation, analogues showing a LAD-like binding mode (Figure 3; see also Figure S3A) tend to have higher docking scores. Interestingly, both **M-26** and **M-37** display a LAD-like binding mode in PfKRS-2, whereas in HsKRS they have either an adenine-like or a lysine-like binding mode, respectively (Figure S3A). These diverse binding modes are due to differences in the sizes of the active site cavities of the two enzymes. Whereas the PfKRS-2 cavity can accommodate the inhibitors and maintain recognition of both the lysine and adenine moieties, the catalytic cavity of HsKRS cannot accommodate both moieties of these compounds at the same time.

Discussion and Conclusions

In recent years cell-based screening has been presented as an attractive way to find new leads for malaria drug development. However, although these approaches are capable of identifying large numbers of hits, they also have serious limitations. If an initial hit is not suitable for chemical modification, for instance, or its target is not known, there might be no opportunity to proceed to hit-to-lead optimization. In this regard, the initial validation of targets based on chemoinformatic predictions can be a useful approach.

Aminoacyl-tRNA synthetases (ARSs) have been recognized for decades as useful targets for drug design.[42,43] Indeed, ARSs continue to be used as targets in antibacterial and antiparasitic drug discovery programs[34,44,45] and have recently received significant attention as promising targets for the development of next-generation antimalarials.[24,38,46,47] Targeting the ARS of a microorganism without inhibiting the human counterpart, however, remains a major challenge. The use of methods for phylogenetic inference helps in recognition of targets with evolutionary histories that might favor the identification of selective compounds. Remarkably, apicomplexan protozoa have incorporated a second form of KRS during the endosymbiotic event that gave rise to apicoplasts. This is evident from the phylogenetic positions assigned in our trees to the apicoplast KRSs of *P. falciparum* and *P. yoelii* (Figure 1C). Whether the apicoplast ancestor was a green or a red alga is still a matter of debate,[11,15] but our data indicate that some of the genes acquired from the apicoplast genome by *Plasmodium* could be direct descendants of bacterial symbionts.

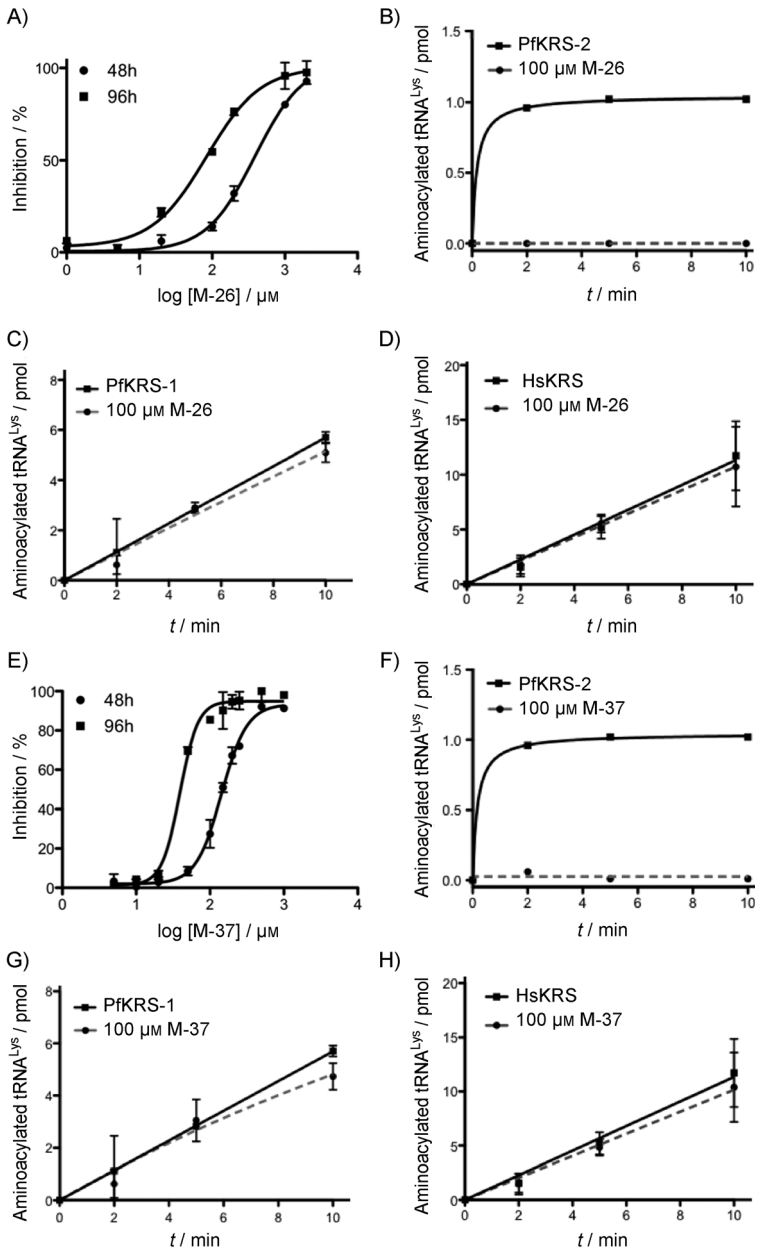


Figure 2. In vitro inhibition of the aminoacylation reactions catalyzed by PfKRS-2, PfKRS-1, and HsKRS. The IC_{50} values for the two most promising inhibitors—**M-26** (A) and **M-37** (E)—were computed both at 48 (●) and at 96 h (■). Both inhibitors show the clear delayed-inhibition effects typical of apicoplastic inhibitors. To verify the target of these inhibitors, we checked their effects on the aminoacylation reactions catalyzed not only by PfKRS-2 (B and F), but also with PfKRS-1 (C and G) and HsKRS (D and H). As can be seen, both compounds inhibit PfKRS-2, whereas the aminoacylation activities of HsKRS and PfKRS-1 remain practically unaffected.

Both our structural and evolutionary data strongly point to PfKRS-2 as a promising target for the development of inhibitors of apicoplast metabolism in apicomplexan organisms, given that they are only distantly related to its human counterparts. Here we demonstrate that this enzyme can indeed be specifically inhibited. Using a combination of computational and experimental approaches, we have built a series of lysyl-adenylate analogue inhibitors designed to be effective against apicoplastic lysyl-tRNA synthetases. We show that some of these molecules inhibit PfKRS-2, whereas the activity of its human homologue remains unaffected by these compounds. Finally, through in silico predictions, we have identified PfKRS-2-specific features of the active site that can explain the selectivities of these compounds. The two compounds described here strongly and specifically inhibit the aminoacylation reaction catalyzed by the PfKRS-2, demonstrating the potential of apicoplastic lysyl-tRNA synthetases as a druggable enzyme targets that can be selectively inhibited and should be further explored for the development of new antimalarial chemotherapies.

Thanks to their cyanobacterial origin, apicoplastic proteins are interesting targets with potential to aid the development of selective drugs. Multiple commonly used antimalarials (e.g., doxycycline, clindamycin, and azithromycin) exert their effects by interfering with apicoplastic proteins. Although apicoplastic proteins might not be the most promising targets for acute malaria treatment, they are nevertheless useful for the development of prophylactic compounds, which in turn are critical for the eradication of malaria.[15,16]

Experimental Section

Homology modeling of PfKRS-2: The sequence of the apicoplastic lysyl-tRNA synthetase of *P. falciparum* was retrieved from the PlasmoDB database (http://PlasmoDB.org). A PSI-BLAST[48] search was performed against the Uniprot database (http://www.uniprot.org) in order to obtain a Position-Specific Scoring Matrix, which was used as input to perform a new BLAST search against the PDB database (http://www.rscb.org), to afford a list of candidate templates to build the model (1lyl, 1bbu, 1e1o). The templates were structurally aligned with the aid of STAMP[49] to create a profile by use of HMMER,[50] which was introduced as meta-template for alignment with the target sequence. Finally, the 9v5 version of MODELLER was employed to create structural models with use of default options.[51] The models generated were manually refined by several methods, including corrections of the alignment with use of the PSI-PRED[52] secondary structure predictions as guideline, and then followed by a new rebuilding of the model. The final model was analyzed with ProSA[53] and validated by using PROCHECK.[54]

Virtual screening and docking: Ligand screening and docking was performed with Glide 5.0.[40] Ligands were prepared such that several conformations were generated for each input ligand, with use of the LigPrep[55] facility of MAESTRO,[56] whereas the setting-up of the proteins (PfKRS-2 and HsKRS) was carried out with the Protein Preparation Wizard facility. The receptor grid defining the docking universe was defined by means of a cubic box centered on the lysyl-adenylate. Schrödinger's GlideScore scoring function was used to score the poses.

Solid-phase synthesis: Each solid-phase synthesis was carried out manually in a polypropylene syringe fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suc-

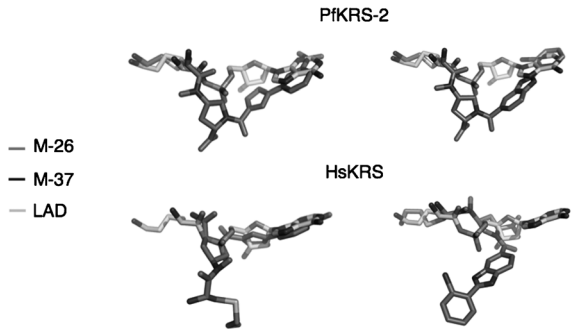


Figure 3. Structural analysis of **M-26** and **M-37** binding modes. In the top panel the binding modes of **M-26** (left) and **M-37** (right) docked into PfKRS-2 are shown. The natural ligand (lysyl-adenylate; LAD) is shown in pale gray. Both compounds have a lysyl-adenylate-like binding mode in the *P. falciparum* binding site (GlideScores of −11.99 and −10.54 for **M-26** and **M-37**, respectively). In the lower panel the binding modes of **M-26** (left) and **M-37** (right) docked into HsKRS are shown. **M-26** has an adenine-like binding mode in *H. sapiens* (GlideScore = −10.85), whereas **M-37** has a lysine-like binding mode (GlideScore = −8.23).

tion. Peptide synthesis for this work employed a combined Boc/Alloc solid-phase strategy on an Fmoc-Rink-Amide-MBHA resin. Washing between deprotection, coupling, and subsequent deprotection steps was carried out with DMF (5×1 min) and CH_2Cl_2 (5×1 min) with 10 mL of solvent per g of resin each time. All the couplings and Fmoc removal were monitored by the Kaiser test. See also Figures S4 and S5 and the supplementary methods.

Synthesis in solution: Unlike in the solid-phase synthesis, the amine protecting group used was now the UV-active *p*-nitrobenzyl carbamate (PNZ), which is more stable under acidic conditions and more readily cleaved by hydrogenolysis than the related benzyl carbamate (Z). Straightforward introduction of the PNZ protecting group followed by conversion of the carboxylic acid into the corresponding primary amide, under standard peptide coupling conditions, gave **60** in good yield. Subsequently, lysine or thialysine were introduced by means of a Mitsonubu reaction. Separation of the formed triphenylphosphine oxide from the corresponding products was laborious. In spite of intensive attempts, complete removal of the triphenylphosphine oxide from the desired product could not be achieved. It was decided to continue the synthesis with the mixture, because the triphenylphosphine oxide should not interfere with the outcomes of the subsequent reactions. Deprotection of the proline derivative by hydrogenolysis (Pd/C under H_2), followed by coupling of the corresponding carboxylic acids under standard peptide coupling conditions (WSC·HCl/HOBt·H_2O), gave products **65**–**69** (Scheme 2) in good yields. Full deprotection of **65**–**69** with trifluoroacetic acid (40%) in CH_2Cl_2 and immediate purification of the crude products by preparative HPLC gave the final products (**M-12**, **M-24**, **M-26**, **M-33**, and **M-37**) in good yields. The final products were characterized by standard techniques such as ^1H and ^{13}C NMR (Figure S5) and exact mass and HPLC-MS.

Phylogenetic analysis of lysyl-tRNA synthetases: The sequences of lysyl-tRNA synthetases reported here are available in Uniprot.[57] We applied the method of structure-based alignment of the active sites of the enzymes, as described elsewhere.[58] Archaeal LysRS-II sequences were initially included in our analysis, but were later dropped for two reasons: 1) they are generally believed to have emerged through lateral gene transfer events,[59–62] and 2) in our initial analyses they clustered consistently within the bacterial clade. Phylogenetic distributions were calculated by distance and maximum likelihood methods with use of the PHYLIP 3.68 package,[63] with 1000 bootstrap replicates in the distance calculations and 100 bootstrap replicates for the maximum likelihood trees.

Cloning and expression of *P. falciparum* PfKRS-2: Soluble and active PfKRS-2 was obtained by the following procedure: the nucleotide sequence of the gene coding for PfKRS-2 without the predicted bipartite signal sequence (ΔPfKRS-2) was codon-optimized for *E. coli*, synthesized (MrGene), and inserted into the plasmid pQE70. Expression screening was carried out with the In-Fusion based Vector Suite at the IRB Protein Expression Core Facility. Different tagged ΔPfKRS-2 constructions were built and inserted into both *E. coli* Rosetta and B834 strains. Three soluble proteins were finally selected for activity assays: these were ΔPfKRS-2-Sumo, ΔPfKRS-2-His, and ΔPfKRS-2-Z, which contained a C-terminal Sumo tag,[64] a N-terminal His tag, and a C-terminal Z tag,[65] respectively. The final concentrations of the His tag, Sumo-His tag, and Z tag enzymes were 3.2, 2.7, and 3.4 μM, respectively. Final aminoacylation assays were performed with ΔPfKRS-2-His.

Pyrophosphate exchange assay: The pyrophosphate (PP_i) exchange assay was used to measure the amino acid activation activities of the three soluble PfKRS-2 recombinant proteins (Figure S6). This assay measures the rate of isotopic exchange of PP_i into ATP, and is therefore focused on the first step of the aminoacylation reaction.[66] Enzyme (5 μL, 2 μM) was added to the reaction mix (2-(4-(2-hydroxyethyl)piperazin-1-yl)ethanesulfonic acid (HEPES)/NaOH (pH 7.2, 50 mM), $MgCl_2$ (20 mM), dithiothreitol (DTT, 1 mM), ATP (2 mM), lysine (4 mM), $NaPP_i$ (2 mM), [^{32}P]$NaPP_i$ (2 MBq μmol^{-1})), and aliquots (22 μL) were withdrawn at 2, 10, and 30 min time points into quenching solution [250 μL, perchloric acid (3.5%, *w/v*), activated charcoal (3%, *w/v*), $NaPP_i$ (100 mM)]. The quenched aliquots were filtered (Whatmann 25 mm filters), and washed once with washing solution [perchloric acid (1.4%, *w/v*), $NaPP_i$ (40 mM)], once with Milli-Q water, and finally with ethanol (100%). The filters were placed into scintillation liquid and counted by the manufacturer's protocol for ^{32}P detection with QuantaSmart Operating Software.

In vitro aminoacylation assays: To characterize the activities of our compounds, their effects on the lysylation of $tRNA^{Lys}$ by ΔPfKRS-2-His were tested. In-vitro-transcribed *P. falciparum* $tRNA^{Lys}$ was prepared as described.[67] Aminoacylation was performed at 37 °C in Hepes/KOH (pH 7.2, 100 mM), aqueous KCl (20 mM), aqueous $MgCl_2$ (30 mM), aqueous DTT (0.5 mM), ATP (5 mM), bovine serum albumin (BSA, 0.1 mg mL^{-1}), [^{3}H]lysine (20 μM, 500 Ci mol^{-1}, PerkinElmer), and in-vitro-transcribed tRNA (5 μM). Reaction aliquots were spotted on 3 mm filter disks and washed in aqueous trichloroacetic acid (5%) with aqueous lysine (100 μM). Radioactivity was determined by liquid scintillation counting. Aminoacylation rates of human lysyl-tRNA synthetase (HsKRS) were also determined to check for possible cross-reactivity of PfKRS-2 inhibitors towards the human homologue. The reactions were performed by addition of in-vitro-transcribed *H. sapiens* $tRNA^{Lys}$ to human HEK 293T cell extracts (5 μL) under the same conditions as for PfKRS-2. Similarly, aminoacylation rates of *P. falciparum* cytosolic lysyl-tRNA synthetase (PfKRS-1) were determined by addition of in-vitro-transcribed nuclear-encoded $tRNA^{Lys}$ of *P. falciparum* to plasmodial extracts (5 μL). Additional controls to test the specificities of the compounds were performed against the tryptophanyl-tRNA synthetase of *P. falciparum*. We used recombinant *P. falciparum* tryptophanyl-tRNA synthetase (a kind gift from Amit Sharma) and in-vitro-transcribed *P. falciparum* $tRNA^{Trp}$ for this purpose. Aminoacylation reactions were performed under the same conditions as described above.

Cell-based drug inhibition assays

LDH activity assay: Initial screens to test the activities of the compounds on 3D7 *P. falciparum* cultures were performed through the LDH activity assay, as previously described.[68] Smears were also prepared for each drug assay to confirm the absorbance results visually. For each tested compound, LDH activity was measured both at 48 and at 96 h in order to check for delayed effects. Mupirocin[24] and borrelidin[25] were used as positive controls of inhibition.[23,24,68]

Fluorescence-assisted cell sorting (FACS): FACS was used to calculate the IC_{50} values of the most active compounds on 3D7 *P. falciparum* cultures. For FACS analysis, Syto-11 was used to discriminate parasitized from nonparasitized red blood cells (RBCs). Each sample was diluted at 1:100 in phosphate-buffered saline (PBS), and Syto-11 in DMSO (0.5 mM) was added to a final concentration of 0.5 μM. Samples were excited at 488 nm and analyzed by using an FC500 flow cytometer. Inhibition rates were measured at several concentrations of the tested compounds. GraphPad Prism version 5.0 (GraphPad Software, San Diego, California, USA) was used to fit the measured inhibitory activities of the compounds to dose-response curves and to infer their corresponding IC_{50} values both at 48 h and at 96 h after drug treatment.

Subcellular localization of PfKRS-2 by immunofluorescence: Apicoplastic subcellular localization of PfKRS-2 was predicted with the aid of different algorithms, including PlasmoAP,[11] PATS,[69] PlasMit,[70] PSORT,[71] and SignalP.[72] To validate these predictions experimentally, the PfKRS-2 leader sequence was inserted into the XhoI/XmaI digested pGlux.1 vector[73] (a kind gift from Alan Cowman), to generate a C-terminal GFP fusion to the N-terminal region of PfKRS-2 that contains the predicted apicoplastic localization signal of the protein.[11] Synchronized cultures of *P. falciparum* 3D7A were electroporated and transfected with the PfKRS-2_{leader}-GFP-containing vector. After 24 h of growth, WR99210 was added to a final concentration of 10 nM to select for transfected parasites.[73] RBCs containing transfected parasites expressing PfKRS-2-GFP were washed and fixed for 5 min in methanol/acetone (90:10) and incubated with anti-ACP primary antibody (kindly provided by Dr. Geoff McFadden), which was used to check for colocalization in the apicoplast. Anti-ACP antibodies were detected with a secondary fluorescent antibody (AlexaFluor 555 mouse anti-rabbit, Invitrogen). The samples were mounted with Mowiol (Calbiochem, Merck Chemicals), and analyzed with a Leica SP2 confocal microscope.

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Keywords: aminoacyl-tRNA synthetases · combinatorial chemistry · drug design · enzyme inhibitors · malaria · molecular modeling

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