Lysyl-tRNA synthetase as a drug target in malaria and cryptosporidiosis

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Malaria and cryptosporidiosis, caused by apicomplexan parasites, remain major drivers of global child mortality. New drugs for the treatment of malaria and cryptosporidiosis, in particular, are of high priority; however, there are few chemically validated targets. The natural product cladosporin is active against blood- and liver-stage Plasmodium falciparum and Cryptosporidium parvum in cell-culture studies. Target deconvolution in P. falciparum has shown that cladosporin inhibits lysyl-tRNA synthetase (PKRS1). Here, we report the identification of a series of selective inhibitors of apicomplexan KRSs. Following a biochemical screen, a small-molecule hit was identified and then optimized by using a structure-based approach, supported by structures of both PKRS1 and C. parvum KRS (CpPKRS). In vivo proof of concept was established in an SCID mouse model of malaria, after oral administration (ED₉₀ = 1.5 mg/kg, once a day for 4 d). Furthermore, we successfully identified an opportunity for pathogen hopping based on the structural homology between PKRS1 and CpPKRS. This series of compounds inhibited CpPKRS and C. parvum and Cryptosporidium hominis in culture, and our lead compound shows oral efficacy in two cryptosporidiosis mouse models. X-ray crystallography and molecular dynamics simulations have provided a model to rationalize the selectivity of our compounds for PKRS1 and CpPKRS vs. (human) HsKRS. Our work validates apicomplexan KRSs as promising targets for the development of drugs for malaria and cryptosporidiosis.

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malaria | cryptosporidiosis | tRNA synthetase

Malaria is caused by Plasmodium spp.; the most significant species from a disease perspective are Plasmodium falciparum and Plasmodium vivax. In 2017, there were estimated to be 219 million clinical cases of malaria and 435,000 deaths from the disease (1, 2). There is an urgent need for new drugs for malaria to deal with the constant threat of drug resistance and to provide new drugs for chemoprophylaxis, prevention of
Significance

Malaria and cryptosporidiosis are major burdens to both global health and economic development in many countries. Malaria caused >400,000 deaths in 2017, and cryptosporidiosis is estimated to cause >200,000 deaths a year. The spread of drug resistance is a growing concern for malaria treatment, and there is no effective treatment for malnourished or immunocompromised children infected with Cryptosporidium. New treatments with novel mechanisms of action are needed for both diseases. We present a selective inhibitor of both Plasmodium and Cryptosporidium lysyl-tRNA synthetase capable of clearing parasites from mouse models of malaria and cryptosporidiosis infection. This provides very strong validation of lysyl-tRNA synthetase as a drug target in these organisms and a lead for further drug discovery.

Transmission, and treatment of relapsing (vivax) malaria (2). In humans, cryptosporidiosis is predominantly caused by Cryptosporidium hominis and Cryptosporidium parvum. The recent Global Enteric Multicenter Study has highlighted cryptosporidiosis as a leading cause of moderate-to-severe diarrheal diseases in infants. The association of cryptosporidiosis with death was the highest for any pathogen in 6- to 18-mo-old children with moderate-to-severe diarrhea (3–5). Cryptosporidiosis is estimated to lead to >200,000 deaths a year and is also associated with malnutrition, stunted growth, and cognitive-development problems in children (6). The currently approved drug nitazoxanide has poor efficacy, particularly in the case of immune-compromised patients and malnourished children, where there is no effective treatment (7, 8).

In the last decade, Plasmodium aminoacyl-tRNA synthetases have received increased attention as new targets for antimalarial drug discovery (9). Aminoacyl-tRNA synthetases catalyze aminoacylation of tRNAs with their cognate amino acids in two stages (10). First, the amino acid is activated by ATP to yield the AMP-activated amino acid, with loss of pyrophosphate, followed by transfer of the amino acid onto the tRNA. By way of example, a series of novel antimalarial bicyclic azetidines, identified by phenotypic screening, were found to inhibit cytosolic Plasmodium phenylalanyl-tRNA synthetase (11). These compounds showed activity across multiple life stages of the parasite and in vivo efficacy in a malaria mouse model. Malaria parasite genomes encode the different lysyl-tRNA synthetases (KRSs) that play a role in translation in either the cytoplasm (PfKRS1) or in the apicoplast (PfKRS2) (9, 12, 13), while Cryptosporidium parasites and humans encode one copy. Human KRS (HsKRS) is found in both the cytosol and mitochondrion and has additional roles within human cells (14).

Hoepfner et al. (15) discovered that the fungal secondary metabolite cladosporin (1) (Fig. 1) was a nanomolar inhibitor of parasite growth in both blood and liver stages of Plasmodium. They demonstrated that cladosporin inhibited cytosolic PfKRS1 with >100-fold selectivity compared with HsKRS. Unfortunately, cladosporin is not amenable to development as a drug lead itself because of high metabolic instability (see data we generated in Fig. 1), which would mean that it would not be significantly orally bioavailable. In this work, we report the discovery and optimization of drug-like inhibitors against PfKRS1 and CpKRS, which showed oral activity in mouse models of malaria and cryptosporidiosis.

Results and Discussion

Characterization of KRS Enzymes. We produced recombinant PfKRS1 (77–583 and 80–583), CpKRS (46-end), and HsKRS (full-length) proteins and developed biochemical assays based on the luciferase ATP consumption assay (Kinase-Glo; Promega) (16), which was suitable for the high-throughput screening, and the pyrophosphate generation assay (EnzChek) (17) format for kinetic characterization of the enzymes. The activities of recombinant enzymes were analyzed by monitoring only the first stage of the aminoacylation reaction. This reaction is suitable for high-throughput screening campaigns and makes the reaction more amenable for steady-state kinetic studies. By using the EnzChek assay, which monitors the production of pyrophosphate (when coupled with pyrophosphatase), Km values were obtained for PfKRS1, CpKRS, and HsKRS for ATP and l-lysine (Table 1 and SI Appendix, Fig. S1 and Table S1). The Km values for the human enzyme are significantly smaller than for the parasite enzymes. This may result from structural differences (see below) between the active sites of the parasite and human enzymes. Even so, the Km values for ATP and l-lysine obtained are comparable with KRSs from other species (https://www.brenda-enzymes.org) (18).

Hit and Lead Discovery. By using the luciferase ATP consumption (Kinase-Glo) assay platform with sub-Km substrate concentrations (thus biasing the assay toward identifying ATP-competitive inhibitors), the GlaxoSmithKline malaria active set of ~13,000 compounds (the Tres Cantos Antimalarial Set) (19) was screened against recombinant PfKRS1, leading to the discovery of a PfKRS1 inhibitor, compound 2 (Fig. 1). Compound 2 displayed similar levels of inhibition of PfKRS1 and growth of P. falciparum to cladosporin. Compound 2 suffered from high metabolic instability (C14 > 50 mM·min−1·g−1 in mouse liver microsomes); however, in contrast to cladosporin, it was chemically tractable.

In the published structure of cladosporin bound to PfKRS1, cladosporin binds within the ATP binding pocket (20). The isocoumarin moiety occupies the same space as the adenine ring of ATP, and the pyran ring occupies the same position as the ribose ring of ATP. The two phenolic hydroxy groups of the isocoumarin ring form hydrogen bonds with the side chain of E332 and the backbone NH of N339, while the carbonyl interacts with a highly coordinated conserved water molecule (20). Screening hit 2 was co-crystalized with PfKRS1 and also binds in the ATP binding pocket (Fig. 2A), in a similar fashion to cladosporin, although the bicyclic core is rotated 30° with respect to cladosporin. The chromosome core stacks between the side chain of F342 on one face and the side chains of H338 and R559 on the other. The ring carbonyl forms an H-bond to the backbone NH of N339, mimicking the N1 of adenine and the O1 OH of cladosporin. The amide carbon H-bonds to a highly conserved water molecule coordinated by the side chain of D558 and the backbone NHs of D558 and R559. This pocket is completed by the substrate lysine and is similar to that occupied by the pyran ring of cladosporin, except that the cyclohexyl ring prope deeps into the pocket (Fig. 2A).

Metabolic-identification studies suggested that hydroxylation occurred in both the phenyl ring of the chromone and the cyclohexyl
ring. By preparing several potential metabolites, we identified the major site of hydroxylation as carbon-6 at the phenyl ring. Addition of a fluorine in the phenyl ring at C-6 blocked hydroxylation at the phenyl ring of the chromone (compound 3; Fig. 1), and introduction of a hydroxyl at the bridgehead of the cyclohexyl substituent was tolerated without loss of potency, while reducing lipophilicity and intrinsic clearance (compound 4). The cocomplex of 4 bound to PFKR5 showed that the native hydrogen bonding key H-bonds from the core scaffold to the protein. The addition of the 6F atom did not afford new interactions with the protein or ordered solvent. The bridgehead hydroxyl was close to the side chain of E500, forming a weak interaction (3.4 Å) and interacting with the ordered water network (SI Appendix, Fig. S7). Addition of fluorines on the 4-position of the cyclohexyl ring in 5 was tolerated and led to excellent metabolic stability, both in mouse and human liver microsomes. (Fig. 1B and Table 2). The complex of 5 bound to PFKR5 showed that the addition of the difluoro moiety on the cyclohexyl ring had minimal effect upon the position of the ligand within the binding site with respect to 4, and there was no evidence of protein rearrangement. In this complex, all polar interactions were retained, although the H-bond between the bridgehead OH and the side chain of Ghu-500 had shortened to 3.0 Å (Fig. 2B).

Enzymatic studies of the inhibition of PFKR5 by compound 5 were performed by using the pyrophosphate generation (EnzCheck) platform. In the presence of saturating concentrations of both substrates, an IC50 of 210 nM was obtained (SI Appendix, Fig. S2A). To study the mechanism of inhibition by compound 5, single-inhibition measurements were performed at a fixed saturating concentration of one substrate and fixed variable concentrations of the second substrate. Under our experimental conditions, results showed a linear competitive inhibition vs. ATP with a Ki of 32 nM and a linear uncompetitive inhibition vs. l-lysine with a Ki of 212 nM (SI Appendix, Fig. S2 B and C and Table S4). These results indicate that compound 5 competes with ATP for the same binding site and only binds in the presence of l-lysine, also suggesting a sequential ordered single kinetic mechanism where l-lysine is the first substrate to bind. The results also show that, in the presence of high concentrations of ATP, the binding affinity of compound 5 is reduced, whereas in the presence of high concentrations of l-lysine, it is increased. Because the mode of inhibition studies are performed at saturating concentration of the cosubstrate, this leads to a lower, more potent Ki against ATP (l-lysine is saturating) and a higher, less potent Ki against l-lysine (ATP is saturating). It is noteworthy that the selectivity ratio for PFKR5/PFKR1 (120-fold in Kinase-Glo) is similar to the 180-fold cellular selectivity observed between P. falciparum parasites and human HepG2 cells.

It was reported that cladosporin binds to PFKR5 in a cooperative manner with l-lysine, leading to significant thermal stabilization (increased melting temperature, Tm) (21). Notably, this stabilization effect was not observed in the human counterpart. To determine whether the chromone series retained a similar stabilization effect, KRS enzymes were incubated with inhibitor and substrates (ATP and l-lysine) in various combinations and gradually heated for observable shifts in Tm. For both PFKR5 and CpKRS enzymes, a marked shift (>2 °C) was observed when l-lysine was present, suggesting an analogous codendent binding mode (Fig. 3). This agrees with the results of the study of the mechanism of inhibition of PFKR5 by compound 5, in which there is a higher Ki determined for this compound in the presence of l-lysine. In contrast, the HsKRS exhibited a reduction in Tm in the presence of inhibitor and l-lysine.

Table 1. Kinetic parameters for KRS determined by using EnzCheck

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Km (ATP)</th>
<th>μM</th>
<th>Hill (ATP)</th>
<th>Vmax (ATP)</th>
<th>μM</th>
<th>Hill (Lys)</th>
<th>Vmax (Lys)</th>
<th>μM</th>
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<tbody>
<tr>
<td>PFKR5</td>
<td>68 ± 3</td>
<td>—</td>
<td>—</td>
<td>413 ± 37</td>
<td>0.89 ± 0.04</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CpKRS</td>
<td>346 ± 128</td>
<td>0.71 ± 0.09</td>
<td>1,045 ± 640</td>
<td>0.49 ± 0.06</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>HsKRS</td>
<td>2.22 ± 0.44</td>
<td>—</td>
<td>—</td>
<td>1.92 ± 0.37</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
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</table>

* Determined in the presence of saturating concentration of the cosubstrate l-lysine, 5 mM l-lysine for Pf and Cp and 0.075 mM for HsKRS.
* Determined in the presence of saturating concentration of the cosubstrate ATP, 0.5 mM ATP for Pf, 2 mM for Cp, and 0.1 mM for HsKRS.

Lead compound 5 showed good systemic exposure after oral dosing with excellent oral bioavailability (F = 100%) and moderate half-life (T1/2 = 2.5 h) (Table 2). A preliminary selectivity study in a 44 receptor-enzyme panel showed no activity at a concentration of 10 μM. The compound did not show inhibition of a range of cytochrome P450 enzymes and did not inhibit hERG (EC50 > 100 μM). While the compound has a good profile in in vitro assays, compound 5 showed toxicity in mice at higher doses (50 mg/kg orally) and was itself not suitable for further progression. It is likely that the toxicity at higher doses is due to inhibition of mammalian KRS. Indeed, at a dose of 50 mg/kg, the blood concentration of compound 5 in mice reached the EC50 for HepG2 cells. Nonetheless, this compound is a drug-like tool compound to explore KRSs as drug targets.

**Profile in Malaria.** Compound 5 was active against both PFKR5 (IC50 = 0.015 μM) and whole-cell bloodstream *P. falciparum* 3D7 (EC50 = 0.27 μM) and was selective compared with both the HsKRS (IC50 = 1.8 μM) and HepG2 cells (EC50 = 49 μM). The drop-off from enzyme to cell is probably due to multiple factors—but the two most likely are that high levels of enzyme inhibition may be required for a phenotypic response and the 1,000-fold increase in the concentration of ATP between the enzyme assay and within the parasite cell (22), given that ATP competes for binding with our inhibitors. The activity of 5 against parasites resistant to chloroquine (PfKL1 (EC50 = 0.51 μM) or atovaquone P(TM90C2B) (EC50 = 0.52 μM) is similar to the drug-sensitive strain (EC50 = 0.39 μM). We investigated the activity against different life-cycle stages of malaria (Table 2). The lead compound 5 showed comparable activity in liver schizonts (P. vivax liver schizont EC50 = 0.95 μM) to sexual blood stages. The in vitro parasite reduction ratio (PRR) assay (23) identified 5 as a compound with a slow rate of killing, displaying an overall biological profile similar to other *Plasmodium* protein-synthesis inhibitors acting on cytosolic targets and to atovaquone (24) (SI Appendix, Fig. S3).

Fig. 2. Binding modes of ligands bound to PFKR1 and CpKRS. (A) PFKR5: Lys2 showing the binding mode of 2 (C atoms, gold) bound to the ATP site of PFKR5 (PDB ID code 6AGT) superimposed upon PFKR1:Lys:cladosporin (cladosporin C atoms, slate; PDB ID code 4PG3). (B) PFKR5:15 showing binding mode of 5 bound to PFKR5 (PDB ID code 6HCU). (C) Overlay of PFKR5:Lys:cladosporin (C atoms, gold; PDB ID code 4PG3) compared with PFKR1:Lys:cladosporin (C atoms, gray; PDB ID code 4PG3). Nonconserved residues within the ligand binding site are labeled. (D) CpKRS:Lys:5 showing binding mode of 5 (C atoms, gold) in complex with CpKRS:Lys (C atoms, gray; PDB ID code 6HCW). H-bonds are shown as dashed lines, and key residues are labeled for clarity.
The biological and pharmacokinetic profile was sufficient to justify a rodent efficacy study. Compound 5 was evaluated in vivo against *P. falciparum* parasites grown in the peripheral blood of NODscidIL2Rγnull mice (SCID), entwined with human erythrocytes (25). Three days after infection, mice were dosed orally once a day for 4 d with 5, at concentrations up to 40 mg/kg (Fig. 4A). From dose–response studies, a daily oral dose of ED₅₀ = 1.5 mg·kg⁻¹ (1.0–2.5 mg·kg⁻¹) (Fig. 4C) or its equivalent estimated daily exposure in blood AUC₀–ED₅₀ = 11,000 ng·h·mL⁻¹·d⁻¹ (6,900–14,000 ng·h·mL⁻¹·d⁻¹) (Fig. 4D), reduced parasitemia by 90% at day 5 of the study. The rate of parasite clearance in vivo is consistent with the PRR data in vitro.

**Pathogen Hopping: Cryptosporidiosis.** There is a high level of sequence identity within the active-site region of PfKRS1 and CpKRS (96% identity) and an overall sequence identity of 47.7% and similarity of 64.6% across the entire protein. Furthermore, structurally, the active sites are very similar. Therefore, we tested cladosporin, the screening hit 2, and the lead compound 5 in a cellular assay against *C. parvum*. The three compounds showed inhibition of parasite growth with EC₅₀ ≤ 1.3 μM (C. parvum) > μM (Iowa strain) (EC₅₀). Subsequently, a crystal structure was obtained for several compounds with KRS:ligand structures were compared with the KRS1 structure and the high ligand binding energy also showed very similar results (Fig. S5). The comparison of PfKRS1 and CpKRS predicted a similar binding mode of compound 5 to that in PfKRS1 and the per-residue contributions to the ligand binding energy also showed very similar results (SI Appendix, Fig. S5 C and E). However, despite the overall high sequence and structural similarity between PfKRS1 and HsKRS, two non-conserved residues were present within the active site: V328 and A309. Further CpKRS:ligand structures were obtained for several compounds within the chromosome series, including CpKRS:5 (Fig. 2D), showing 5 to bind in an identical manner to CpKRS as to PfKRS1. In cryptosporidiosis, the parasite is found predominantly in the epithelial cells (enterocytes) in the gastrointestinal tract (8), although it is thought that there may also be some parasites present in the biliary tract. Therefore, it is likely that a compound used for treating cryptosporidiosis would need to have a good exposure in the gastrointestinal tract and possibly also some systemic exposure (27). After oral dosing, compound 5 was completely bioavailable. However, some compound was present in mouse stools (17% of oral dose), suggesting that some biliary excretion had occurred. This raises the possibility of deliberately utilizing enterohepatic recirculation to maintain both gastrointestinal and systemic exposure. Compound 5 showed in vivo efficacy in two different Cryptosporidium mouse models, the NOD SCID gamma and INF-γ-knockout mouse models. INF-γ-knockout mice (28, 29) were infected orally with *N. Lemurigenic* expressing transgenic *C. parvum* oocysts. Treatment started upon patency 4 d postinfection (p.i.), and mice were treated orally once a day for 7 d. Infection was monitored daily by luciferase measurements in pooled feces of the entire cage. Mice were followed for 3 wk after completion of drug treatment. Compound 5, when dosed orally at 20 mg/kg once a day for 7 d, reduced parasite shedding below detection level, and this reduction was sustained for 3 wk after treatment had stopped (Fig. 4E). NOD SCID gamma mice were infected with *C. parvum* oocysts (26). Treatment started 7 d.p.i., and mice were treated orally once a day for 7 d. The study was run with four mice per cage; infection was monitored by quantitative PCR on day eight for individual mice, and data are shown as oocysts per milligram of feces. Compound 5 dosed orally at a concentration of 20 mg/kg once a day for 7 d showed 96% reduction of parasite shedding comparable to paromomycin (Fig. 4F).

**Molecular Basis of PfKRS1 Inhibitor Selectivity.** Molecular dynamics (MD) simulations were successful in reproducing the binding pose and interactions of compound 5 observed in the co-crystal structure of PfKRS1 (SI Appendix, Fig. S5A). In addition to those interactions, the inhibitor was found to be stabilized by hydrophobic contacts established between the cyclohexyl moiety and the bound substrate l-lysine. Simulations performed in the absence of l-lysine showed a notable destabilization of compound 5, suggesting a key role of l-lysine in the binding of PfKRS1 inhibitors (SI Appendix, Fig. S5E). This was confirmed by both the structural information and the thermal shift assays (Figs. 2 and 3).

MD simulations of HsKRS predicted a similar binding mode of compound 5 to that in PfKRS1, and the per-residue contributions to the ligand binding energy also showed very similar results (SI Appendix, Fig. S5 C and E). However, despite the overall high sequence and structural similarity between PfKRS1 and HsKRS, two non-conserved residues were present within the active site: V328 and A309 in PfKRS1 correspond to bulkier residues Q321 and T337 in HsKRS (Fig. 5 A–C). To investigate whether this subtle difference might have an impact on the binding process of compound 5, both enzymes were also simulated in the absence of inhibitor (apo systems), with the main focus placed on the conformational features of the binding pocket. In apo-PfKRS1, due to the smaller size of V328 and A309 side chains, the ligand binding pocket remained accessible to compound 5 (Fig. 5B). Conversely, in apo-HsKRS, the binding site remained partially inaccessible due the extended side chain of Q321, which formed a hydrogen-bond network with R323, T337, and E339 conserved residues were present within the active site: V328 and A309 (Fig. S5). A disruption of the hydrogen-bond interactions within the active site of HsKRS is required to enable the binding of the inhibitor.

The comparison of apo and holo PfKRS1 and HsKRS systems also showed significant differences in the dynamics of residues neighboring the active site. In apo-PfKRS1, R330 was highly flexible and

<table>
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<tr>
<th>Compound</th>
<th>Initial Tₘ (°C)</th>
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<th>5</th>
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<tbody>
<tr>
<td>Pf</td>
<td>Cp</td>
<td>P</td>
<td>Hs</td>
</tr>
<tr>
<td>AQP</td>
<td>45.9</td>
<td>4.5</td>
<td>62.0</td>
</tr>
<tr>
<td>Lys</td>
<td>43.6</td>
<td>5.3</td>
<td>39.5</td>
</tr>
<tr>
<td>AQP+Lys</td>
<td>46.1</td>
<td>7.2</td>
<td>60.2</td>
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Fig. 3. Heatmap showing effects of compounds 1 and 5 on the melting temperature (ΔTₘ) of KRS enzymes.
was stabilized only after binding of the inhibitor. Similar behavior was observed for the loop 282–291, which was highly disordered in the absence of the ligand, but became ordered upon ligand binding (Fig. S8 and SI Appendix, Fig. S6C). This was corroborated by the marked positive shift in PyKRS1’s T_m in the presence of inhibitor and l-lysine compared with the apo state (Fig. 3). On the other hand, in HsKRS, the equivalent R323 and loop 274–282 remained highly stable, regardless of the presence of the ligand (Fig. 5C and SI Appendix, Fig. S6B and C). Such ligand-induced stabilization observed for the mobile loop and residues near the PyKRS1 active site could potentially account for a more favorable binding of compound 5 to PyKRS1 with respect to HsKRS. The CpKRS system exhibited behavior similar to PyKRS1: an accessible binding site and a high degree of flexibility of the loop and R295 in the apo-state and dramatic stabilization upon ligand binding, which, again, was supported by the large observed shift in T_m, from the apo state to the l-lysine plus inhibitor state (Figs. 3 and S5D and SI Appendix, Fig. S6B and C). This provided a rationale for the compound 5 affinity toward CpKRS.

The results of MD simulations suggested that the parasite KRS selectivity vs. HsKRS observed for compound 5 was due to a combination of two factors: (i) a more favorable (i.e., more accessible) configuration of the binding site in the parasite enzyme, and (ii) a higher degree of stabilization for the PyKRS1 and CpKRS residues upon ligand binding. Our findings are in good agreement with previous experimental and structural studies that reported an increased flexibility of PyKRS1 over HsKRS and suggested that the active-site loops in aminocycl-tRNA transferases are likely to have a critical role in specific ligand recognition (21, 30, 31). It is likely that full understanding of the observed selectivity can only be obtained by reproducing the entire process of ligand binding to KRS.

In conclusion, identification of the molecular targets of phenotypic hits and subsequent target-based approaches to these targets is a promising way to develop new antiinfective agents. PyKRS1 was shown to be the target of the natural product cladospisin, which was found to be active against P. falciparum in cell culture. Cladospisin itself is not suitable for progression to animal studies, as it is not metabolically stable or orally bioavailable. Given that cladospisin has a complex synthesis with low overall yield (eight steps with an overall yield of 8%) (32), chemical modification to improve the metabolic stability looked challenging, and a long synthesis means that the cost of goods would likely fall outside the Target Product Profiles for malaria and cryptosporidiosis. Therefore, we carried out a small-molecule screen against PyKRS1 to find an alternate chemotype for optimization. Following optimization of a hit molecule (2), we identified a metabolically stable and orally bioavailable compound (5) which inhibited PyKRS1 selectively. A low oral dose (1.5 mg/kg once a day for 4 d) of our KRS inhibitor, compound 5, reduced parasitemia by 90% in the malaria SCID mouse model. This result was obtained in vivo validation of PyKRS1 as a promising antimalarial target for drug development. We have also successfully undertaken pathogen-hopping (33), demonstrating that this compound series inhibits CpKRS and the growth of C. parvum in vitro. Moreover, compound 5 showed a reduction of parasite burden by two orders of magnitude when dosed orally for 7 d in two different mouse models of cryptosporidiosis. There are very few validated targets for cryptosporidiosis. There are very few validated targets for cryptosporidiosis.