Baragaña, Beatriz; Forte, Barbara; Choi, Ryan; Nakazawa Hewitt, Stephen; Bueren-Calabuig, Juan A; Pisco, João Pedro; Peet, Caroline; Dranow, David M; Robinson, David A; Jansen, Chimed; +67 more... Norcross, Neil R; Vinayak, Sumiti; Anderson, Mark; Brooks, Carrie F; Cooper, Caitlin A; Damerow, Sebastian; Delves, Michael; Dowers, Karen; Duffy, James; Edwards, Thomas E; Hallyburton, Irene; Horst, Benjamin G; Hulverson, Matthew A; Ferguson, Liam; Jiménez-Díaz, María Belén; Jumani, Rajiv S; Lorimer, Donald D; Love, Melissa S; Maher, Steven; Matthews, Holly; McNamara, Case W; Miller, Peter; O’Neill, Sandra; Ojo, Kayode K; Osuna-Cabello, Maria; Pinto, Erika; Post, John; Riley, Jennifer; Rottmann, Matthias; Sanz, Laura M; Scullion, Paul; Sharma, Arvind; Shepherd, Sharon M; Shishikura, Yoko; Simeons, Frederick RC; Stebbins, Erin E; Stojanovski, Laste; Straschil, Ursula; Tamaki, Fabio K; Tamjar, Jevgenia; Torrie, Leah S; Vantaux, Amélie; Wittkowski, Benoît; Wittlin, Sergio; Yogavel, Manickam; Zuccotto, Fabio; Angulo-Barturen, Íñigo; Sinden, Robert; Baum, Jake; Gamo, Francisco-Javier; Mäser, Pascal; Kyle, Dennis E; Winzeler, Elizabeth A; Myler, Peter J; Wyatt, Paul G; Floyd, David; Matthews, David; Sharma, Amit; Striepen, Boris; Huston, Christopher D; Gray, David W; Fairlamb, Alan H; Pisiakov, Andrei V; Walpole, Chris; Read, Kevin D; Van Voorhis, Wesley C; Gilbert, Ian H; (2019) Lysyl-tRNA synthetase as a drug target in malaria and cryptosporidiosis. Proceedings of the National Academy of Sciences of the United States of America, 116 (14). pp. 7015-7020. ISSN 0027-8424 DOI: https://doi.org/10.1073/pnas.1814685116

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


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Edited by Daniel E. Goldberg, Washington University School of Medicine, St. Louis, MO, and accepted by Editorial Board Member Stephen M. Beverley

February 5, 2019 (received for review September 10, 2018)

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 dactosporin is active against blood- and liver-stage Plasmodium falciparum and Cryptosporidium parvum in cell-culture studies. Target deconvolution in P. falciparum has shown that dactosporin 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 (CpKRS). 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 CpKRS. This series of compounds inhibits CpKRS 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 CpKRS vs. (human) HsKRS. Our work validates apicomplexan KRSs as promising targets for the development of drugs for malaria and cryptosporidiosis.

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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.

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 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 the production of pyrophosphate (when coupled with pyrophosphatase). \( K_m \) values were obtained for PfKRS1, CpKRS, and HsKRS for ATP and L-lysine (Table 1 and SI Appendix, Fig. S1 and Table S1). The \( K_m \) values for the human enzyme are significantly smaller than for the parasite enzymes. This may be due to structural differences (see below) between the active sites of the parasite and human enzymes. Even so, the \( K_m \) 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-\( K_m \) 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 1. Compound 2 suffered from high metabolic instability (\( C_L \) > 50 mL/min·g-1 in mouse liver microsomes); however, in contrast to cladosporin 1, 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 system 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-crystallized with PfKRS1 and also binds in the ATP binding pocket (Fig. 2.4), 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. Cladosporin binds in the ATP binding pocket (20). The isocoumarin moiety projects into a pocket formed by the side chains of R330, F342, and S344 and the backbone of L555 and G556. 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 probes deeper into the pocket (Fig. 2.4).

Metabolite-identification studies suggested that hydroxylation occurred in both the phenyl ring of the chrome and the cyclohexyl

EnzChek assay, which monitors the production of pyrophosphate (when coupled with pyrophosphatase). \( K_m \) values were obtained for PfKRS1, CpKRS, and HsKRS for ATP and L-lysine (Table 1 and SI Appendix, Fig. S1 and Table S1). The \( K_m \) values for the human enzyme are significantly smaller than for the parasite enzymes. This may be due to structural differences (see below) between the active sites of the parasite and human enzymes. Even so, the \( K_m \) values for ATP and L-lysine obtained are comparable with KRSs from other species (https://www.brenda-enzymes.org/) (18).

**Fig. 1.** The structure of cladosporin 1, evolution of the lead 5 from the screening hit 2. Enzyme data were obtained with the Kinase-Glo assay. MLM, mouse liver microsomes.

**Fig. 2.** X-ray structures of compound 1 and compound 2 bound to PfKRS1 (PDB: 5nje, 5njk, and 5njo) (18).
Table 1. Kinetic parameters for KRS determined by using EnzCheck

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$, ATP, μM*</th>
<th>Hill$_{ATP}$</th>
<th>$K_m$, Lys, μM</th>
<th>Hill$_{Lys}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKRS1</td>
<td>68 ± 3</td>
<td>—</td>
<td>413 ± 37</td>
<td>0.89 ± 0.04</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>
</tr>
<tr>
<td>HsKRS</td>
<td>2.22 ± 0.44</td>
<td>—</td>
<td>1.92 ± 0.37</td>
<td>—</td>
</tr>
</tbody>
</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.

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 chrome (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 cleavage (compounds 4). The complex of 4 bound to PKRS1 showed that this binding mode was retained (Table S2). 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 PKRS1 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 PKRS1 by compound 5 were performed by using the pyrophosphate generation (EnzCheck) platform. In the presence of saturating concentrations of both substrates, an IC$_{50}$ of 210 mM 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 $K_i$ of 32 mM and a linear uncompetitive inhibition vs. L-lysine with a $K_i$ of 212 mM (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 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 $K_i$ against ATP (L-lysine is saturating) and a higher, less potent $K_i$ against L-lysine (ATP is saturating). It is noteworthy that the selectivity ratio for H$_s$KRS/PKRS1 (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 PKRS1 in a cooperative manner with L-lysine, leading to significant thermal stabilization (increased melting temperature, $T_m$) (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 $T_m$. For both PKRS1 and CpKRS enzymes, a marked shift (>2 °C) was observed when L-lysine was present, suggesting an analogous codependent binding mode (Fig. 3). This agrees with the results of the study of the mechanism of inhibition of PKRS1 by compound 5, in which there is a higher $K_i$ determined for this compound in the presence of L-lysine. In contrast, the H$_s$KRS exhibited a reduction in $T_m$ in the presence of inhibitor and L-lysine.

Lead compound 5 showed good systemic exposure after oral dosing with excellent oral bioavailability (F = 100%) and moderate half-life ($T_{1/2} = 2.5$ h) (Table 2). A preliminary selectivity study in a 44 receptor/transporter 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 (EC$_{50}$ > 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 EC$_{50}$ 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 PKRS1 (IC$_{50}$ = 0.015 μM) and whole-cell bloodstream $P$. falciparum 3D7 (EC$_{50}$ = 0.27 μM) and was selective compared with both the H$_s$KRS (IC$_{50}$ = 1.8 μM) and HepG2 cells (EC$_{50}$ = 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 PK (K1) (EC$_{50}$ = 0.51 μM) or atovaquone Pf (TM90C2B) (EC$_{50}$ = 0.52 μM) is similar to the drug-sensitive strain Pf (NF54) (EC$_{50}$ = 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 EC$_{50}$ = 0.95 μM) to asexual 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 PKRS1 and CpKRS. (A)PKRS1: Lys:2 showing the binding mode of 2 (C atoms, gold) bond to the ATP site of PKRS1 (PDB ID code 6AGT) superimposed upon PKRS1:Lys:cladosporin (cladosporin C atoms, slate; PDB ID code 4P3G). (B)PKRS1:5 showing binding mode of 5 bound to PKRS1 (PDB ID code 6HCU). (C)Overlay of CpKRS:Lys:cladosporin (C atoms, gold; PDB ID code 5ELO) compared with PKRS1:Lys:cladosporin (C atoms, gray; PDB ID code 4P3G). 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.
Table 2. In vitro activity and DMPK profile of lead compound 5

<table>
<thead>
<tr>
<th>Assay</th>
<th>Data</th>
</tr>
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<tbody>
<tr>
<td><em>P. falciparum</em> 3D7 EC\textsubscript{50}</td>
<td>0.27 \mu M</td>
</tr>
<tr>
<td><em>P. vivax</em> liver schizonts/hypnozoites EC\textsubscript{50} (prophylactic mode)</td>
<td>0.95 \mu M/70 \mu M</td>
</tr>
<tr>
<td><em>P. berghei</em> liver schizonts EC\textsubscript{50}</td>
<td>0.9 \mu M</td>
</tr>
<tr>
<td><em>P. falciparum</em> stage V gametocytes EC\textsubscript{50}</td>
<td>83 \mu M</td>
</tr>
<tr>
<td><em>P. falciparum</em> male/female gamete formation EC\textsubscript{50}</td>
<td>&gt;1 \mu M</td>
</tr>
<tr>
<td>FaSSIF solubility</td>
<td>255 \mu M</td>
</tr>
<tr>
<td>Microsomal stability C\textsubscript{int}</td>
<td>1 (mouse), &lt;0.5 (human) \text{mL-min\textsuperscript{-1}·g\textsuperscript{-1}}</td>
</tr>
<tr>
<td>Hepatocyte stability C\textsubscript{int}</td>
<td>0.5 (mouse), &lt;0.5 (human) \text{mL-min\textsuperscript{-1}·g\textsuperscript{-1}}</td>
</tr>
<tr>
<td>CYP inhibition (CYP1A2, 2D6, 2C9, 2C19, 3A4) IC\textsubscript{50}</td>
<td>&gt;10 \mu M</td>
</tr>
<tr>
<td>Mouse PK intravenously (dose, Clb, AUC, T\textsubscript{1/2}, Vdss)</td>
<td>3 mg/kg, 3.4 \text{mL-min\textsuperscript{-1}·g\textsuperscript{-1}}, 890 \mu g·min/mL, 2.5 h, 1 L/kg</td>
</tr>
<tr>
<td>Mouse PK orally (dose, T\textsubscript{max}, AUC\textsubscript{0–1440} F%)</td>
<td>10 mg/kg, 2 h, 5.4 \mu g/mL, 1,300–3,000 \mu g·min/mL, 100%</td>
</tr>
</tbody>
</table>

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), engrafted 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 ED\textsubscript{50} = 1.5 mg·kg\textsuperscript{-1} (1.0–2.3 mg·kg\textsuperscript{-1}) (Fig. 4C), or its equivalent estimated daily exposure in blood AUC\textsubscript{0–24} = 11,000 ng·h·mL\textsuperscript{-1} (6,900–14,000 ng·h·mL\textsuperscript{-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 EC\textsubscript{50} of 0.7, 1.2, and 2.5 \mu M, respectively. Our lead 5 is similarly active against a small panel of isolates: *C. hominis* (TUS02) (EC\textsubscript{50} = 6.0 \mu M) and the *C. parvum* Iowa strain (EC\textsubscript{50} = 1.3 \mu M). In time-kill curve studies conducted by using *C. parvum* in the HCT-8 cell-culture system (26), both cladosporin and compound 5 eliminated parasites at an exponential rate, consistent with other protein synthesis inhibitors studied to date (SI Appendix, Fig. S4). Subsequently, a crystal structure was determined of CpKRS in complex with cladosporin and 1-lysine [Protein Data Bank (PDB) ID code 5SE0] (Fig. 2C), showing retention of the ligand binding mode compared with the PfKRS1 structure and the high level of sequence conservation within the active site, with only two sequence differences at the base of the ligand binding site (PfKRS1 V328 Lint and CpKRS N293 A309). Further CpKRS:ligand structures were obtained for several compounds within the chromone 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 Nluc-ooocysts. Treatment started upon parasite shedding, 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* ooocysts (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 ooocysts 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. S54). In addition to those interactions, the inhibitor was found to be stabilized by hydrophobic contacts established between the cyclohexyl moiety and the bound substrate 1-lysine. Simulations performed in the absence of 1-lysine showed a notable destabilization of compound 5, suggesting a key role of 1-lysine in the binding of PfKRS1 inhibitors (SI Appendix, Fig. S55). 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 S344 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 S344 side chains, the ligand binding pocket remained accessible to compound 5 (Fig. 5B). Conversely, in apo-HsKRS, the binding site remained partially inaccessible due to the extended side chain of Q321, which formed a hydrogen-bond network with R323, T337, and E339 (Fig. 5C and SI Appendix, Fig. S6E). Hence, 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
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 \( \Delta \gamma \text{KRS1}'s \) in the presence of inhibitor and L-lysine compared with the apo state (Fig. 3). On the other hand, in \( \text{HsKRS} \), 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 \( \text{PYKRS1} \) active site could potentially account for a more favorable binding of compound \( 5 \) to \( \text{PYKRS1} \) with respect to \( \text{HsKRS} \). The \( \text{CpKRS} \) system exhibited behavior similar to \( \text{PYKRS1} \): 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 SD and SI Appendix, Fig. S6B and C). This provided a rationale for the compound \( 5 \) affinity toward \( \text{CPKRS} \).

The results of MD simulations suggested that the parasite KRS selectivity vs. \( \text{HsKRS} \) 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 \( \text{PYKRS1} \) and \( \text{CPKRS} \) residues upon ligand binding. Our findings are in good agreement with previous experimental and structural studies that reported an increased flexibility of \( \text{PYKRS1} \) over \( \text{HsKRS} \) and suggested that the active-site loops in aminoacyl-RNA 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. \( \text{PYKRS1} \) was shown to be the target of the natural product cladosporin, which was found to be active against \( \text{P. falciparum} \) in cell culture. Cladosporin itself is not suitable for progression to animal studies, as it is not metabolically stable or orally bioavailable. Given that cladosporin 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 \( \text{PYKRS1} \) 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 \( \text{PYKRS1} \) 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% at day 5 of the experiment. At day 8, there is a 10,000-fold reduction in parasite levels compared with control. Therefore, we identified a metabolically stable and orally bioavailable compound \( 5 \) which inhibited \( \text{PYKRS1} \) 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% at day 5 of the experiment. At day 8, there is a 10,000-fold reduction in parasite levels compared with control.
malaria and cryptosporidiosis in animal models. We have identified a valuable tool compound, although further optimization is required in terms of both potency and selectivity to obtain a preclinical candidate.


Materials and Methods

Full details are in SI Appendix. This includes the following information: (i) the chemical synthesis of compounds described in the paper; (ii) the methods for protein expression and purification, kinetic characterization of enzymes, screening of library, and mode of inhibition studies; (iii) the methods for parasite assays using different life-cycle stages of P. falciparum and different species of Cryptosporidium; (iv) methods for in vitro drug metabolism and pharmacokinetic assay; (v) methods for in vivo pharmacokinetics and efficacy studies; (v) details for molecular modeling and dynamics simulations; (vi) details of X-ray crystallography; (vii) ethical use of animals; and (viii) detailed author contributions. Ethical approval for rodent experiments was given by the University of Vermont Institutional Animal Care and Use Committee, The Art of Discovery Institutional Animal Care and Use Committee (TAD-IACUC), Veterinäramt Basel Stadel, the University of Dundee “Welfare and Ethical Use of Animals Committee,” and the University of Georgia Animal Care and Use Committee. Human biological samples were sourced ethically and used under informed consent.

ACKNOWLEDGMENTS. We thank the European Synchrontron Radiation Facility for beamtime, highlighting the staff of beamlines BM14 and ID29, Diamond Light Source for beamtime (proposal mx10071); the staff of beamline I24 for assistance with crystal testing and data collection; the entire Seattle Structural Genomics Center for Infectious Disease team; the Division of Biological Chemistry and Drug Discovery Protein Production Team; GlassX淋Kline for the Tres Campos (fungal) chemogenomic screening set; the Scottish Blood Transfusion Centre (Ninewells Hospital, Dundee) for providing human erythrocytes; Christoph Fischl and Sibylle Sax at the SwissTPH for technical assistance with the SCID mouse model; and Anja Schäfer for technical assistance with the in vitro amimalarial activity testing. The Art of Discovery thanks Dr. Cristina Equitabl and P. falciparum porcine liver and human tissue (Haldagak, Spain) and the Bank of Blood and Tissues (Barcelona, Spain) for providing human blood. The University of California, San Diego thanks Jenya Antonova-Koch for help. This work was supported by the Bill and Melinda Gates Foundation through Grant OPP1032548 to the Structure-Guided Drug Discovery Coalition and OPP1134302 (to B.S.). This work was also supported in part from federal funds, from the NIHNational Institute of Allergy and Infectious Diseases Grant R21AI123690 (to K.K.O.) and Contracts HHSN27220170002C and HHSN27220170009C (to P.J.M.); Medicines for Malaria Venture (through access to assays to I.H.G. and through RD/08/2800 to J.B.); Wellcome Trust for support of the X-ray Crystallography Facility 094090, IT support Grant 105021 (to I.H.G.), and Institutional Strategic Support Fund 204816 (to A.V.P.), all at the University of Dundee and for Investigator Award 100993 (to J.B.).