Structural basis of malaria parasite phenylalanine tRNA-synthetase inhibition by bicyclic azetidines

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The inhibition of *Plasmodium* cytosolic phenylalanine tRNA-synthetase (cFRS) by a novel series of bicyclic azetidines has shown the potential to prevent malaria transmission, provide prophylaxis, and offer single-dose cure in animal models of malaria. To date, however, the molecular basis of *Plasmodium* cFRS inhibition by bicyclic azetidines has remained unknown. Here, we present structural and biochemical evidence that bicyclic azetidines are competitive inhibitors of L-Phe, one of three substrates required for the cFRS-catalyzed aminoacylation reaction that underpins protein synthesis in the parasite. Critically, our co-crystal structure of a PvcFRS-BRD1389 complex shows that the bicyclic azetidine ligand binds to two distinct sub-sites within the PvcFRS catalytic site. The ligand occupies the L-Phe site along with an auxiliary cavity and traverses past the ATP binding site. Given that BRD1389 recognition residues are conserved amongst apicomplexan FR5s, this work lays a structural framework for the development of drugs against both *Plasmodium* and related apicomplexans.
Malaria, caused by apicomplexan parasites of the genus *Plasmodium*, presents a formidable global health challenge mainly due to the emergence of parasite strains that are resistant to front-line drugs. It is therefore necessary to discover and validate new drug targets as well as compounds whose efficacy is unaffected by mechanisms of resistance to traditional antimalarials. Ideally, an antimalarial development candidate should have a new mechanism of action (MoA) with rapid asexual blood-stage parasite reduction, and activity against all stages of the parasite lifecycle in the human host. Recently, a series of small molecules based on a bicyclic azetidine core have been discovered that exhibit multistage antimalarial activity and can achieve single-dose cures in a mouse model of malaria. It was demonstrated that these compounds exert their antimalarial activity via inhibition of *Plasmodium falciparum* cytosolic phenylalanyl-tRNA synthetase (cFRS), an enzyme essential for protein synthesis. The PfFRS was found to be validated both genetically and biochemically as a drug target for the bicyclic azetidine series of molecules, setting the stage for further drug development efforts. Aminoacyl-tRNA synthetases (aaRSs) activate amino acids as aminoacyl adenylates (AA-AMP), and enable their relay to the 3′-ends of cognate tRNAs as feed for ribosomes. Inhibition of aaRSs therefore results in the interruption of cell growth and ultimately in cell death. Of note, *Plasmodium* aaRSs other than FRS have also recently become the focus of antimalarial development efforts.

As previously shown for *Plasmodium falciparum*, the parasite has three protein translation compartments: in the cytoplasm, apicoplast and in the mitochondria where FRSs reside to feed charged tRNAs into ribosomal-based protein synthesis. In both *P. falciparum* and *P. vivax*, FRSs exist as heterodimers of alpha (α) and beta (β) subunits that further dimerize to form a complex of (αβ)2. This hetero-tetrameric organization of cFRSs is conserved but with significant differences in the chain lengths and functions of α and β subunits. The FRS α subunit contains the active site and catalyzes the two-step aminoacylation reaction, while the main functions of the β subunit are to recognize the anticodon region of tRNA and to edit mischarged tRNA molecules with isosteric amino acids such as tyrosine. The cFRSs are highly conserved and exhibit high sequence identity amongst all stages of the parasite lifecycle in the human host. Recently, a series of small molecules based on a bicyclic azetidine core have been discovered that exhibit multistage antimalarial activity and can achieve single-dose cures in a mouse model of malaria. It was demonstrated that these compounds exert their antimalarial activity via inhibition of *Plasmodium falciparum* cytosolic phenylalanyl-tRNA synthetase (cFRS), an enzyme essential for protein synthesis. The PfFRS was found to be validated both genetically and biochemically as a drug target for the bicyclic azetidine series of molecules, setting the stage for further drug development efforts. Aminoacyl-tRNA synthetases (aaRSs) activate amino acids as aminoacyl adenylates (AA-AMP), and enable their relay to the 3′-ends of cognate tRNAs as feed for ribosomes. Inhibition of aaRSs therefore results in the interruption of cell growth and ultimately in cell death. Of note, *Plasmodium* aaRSs other than FRS have also recently become the focus of antimalarial development efforts.

**Results**

**BRD1389 binds FRS selectively and inhibits aminoacylation via L-Phe competition.** To determine the mode of inhibition of cFRS by bicyclic azetidines, we turned to BRD1389 (Fig. 1a, Supplementary Fig. 1, 2), a series analogue with high in vitro activity. cFRS inhibition by bicyclic azetidines, we turned to BRD1389 (Fig. 1a, Supplementary Fig. 1, 2), a series analogue with high in vitro activity. cFRS inhibition of FRS with BRD1389, a potent antimalarial from the bicyclic azetidine chemical series.

**Overall structure of PvcFRS.** Toward unravelling the structural basis of the protein–inhibitor interaction, we crystallized the PvcFRS-BRD1389 complex and obtained crystals that diffracted to 3 Å. The structure was solved by molecular replacement (MR) using HsFRS as a template (PDB: 3L4G [https://www.rcsb.org/structure/3l4g]). The PvcFRS-BRD1389 crystals belong to orthorhombic space group P212121 with one heterodimer (αβ) per asymmetric unit. The (αβ)2 biological heterotetrameric assembly is completed via the crystallographic two-fold axis along c. The PvcFRS (αβ)2 assembly is consistent with the size exclusion chromatography profile of purified protein, where it elutes at a size of ~298 kDa (Supplementary Fig. 3a). Our final PvcFRS-BRD1389 atomic model contains 905 residues—299α subunit, 606β subunit, one Mg2+ ion, and one ligand (BRD1389). The N-terminal domain (residues 1–270) of the α subunit was absent in the crystal and no electron density was observed for it. This was further verified by crystal packing analysis of PvcFRS-BRD1389 complex and via SDS-PAGE of crystallised protein (Supplementary Fig. 4). The overall fold and organization of α and β subunits is very similar to that of the human orthologue (HscFRS, Fig. 2a, b) with the exception that the PvcFRS β subunit lacks an 18-residue-long fragment within its PB1 domain whereas it has two unique insertions (IL-1: 421–551) in its B2 subdomain (Supplementary Fig. 5). Intriguingly, the association of α1 and β2 subdomains (of the β subunit) with a subunit is significantly different between the parasite and human enzymes (Fig. 1e–j, Supplementary Fig. 6a). In HsFRS, the α subunit is enclosed by β1 and β2 subdomains of the β subunit with a subunit is associated with the α subunit (Fig. 1i, i). This difference arises due to the shorter linker length between β1 and β2 of PvcFRS when compared to the HsFRS (Fig. 1h, k) which has a three residue (384–TYT–386) insertion. Interestingly, this three-residue shorter linker is observed in all human malaria parasites (Fig. 1b, k) and is suggestive of domain swapping (Fig. 1g) in PvcFRS to form a closed, functional hetero-tetrameric (αβ)2 assembly.

**BRD1389 occupies the L-Phe site and an auxiliary site on *Plasmodium* cFRS.** During the refinement of PvcFRS model, an
Fig. 1 BRD1389 binds cFRS selectively and inhibits aminoacylation via L-Phe competition. **a** Chemical structure of BRD1389. **b** Inhibition of the aminoacylation activity of *Pf* (*Plasmodium falciparum*), *Pv* (*Plasmodium vivax*), *Hs* (*Homo sapiens*) and *Pf* mutant (L550V) cFRS enzymes by BRD1389. These assays were performed at concentrations ranging from 100 μM to 0.1 nM and the IC$_{50}$ values were calculated by non-linear regression. Data are shown as mean ± SD (*n* = 3 independent experiments). **c**, **d** Mode of BRD1389 inhibition. BRD1389 is a competitive inhibitor of *Pf* cFRS with respect to L-Phe (K$_{i}$ = 6 nM) while it is likely a non-competitive inhibitor with respect to ATP (K$_{i}$ = 10 nM). Lineweaver-Burk plots were obtained at a saturating concentration of either ATP (500 μM) or L-Phe (1000 μM) with varying concentrations of other substrate: L-Phe (1000–15.6 μM) or ATP (500–15.6 μM) and inhibitor BRD1389 (1 × IC$_{50}$ (blue triangle), 0.5 × IC$_{50}$ (yellow square), and 0 × IC$_{50}$ (black circle) Data are shown as mean ± SD (*n* = 3 independent experiments). The error bars indicate standard deviation (*n* = 3). **e**, **f** Surface view of heterodimeric assembly (αβ) of *Pv*cFRS and *Hs*cFRS (α-subunit in grey and β-subunit in pink) structures. The subdomains of β1 and β2 of β-subunit are marked. **g** Superimposed structure of *Pv/Hs*cFRS displaying movement of subdomain β2 of β-subunit and hence the possible domain swap in the malaria enzyme. **h** Close-up view of β-subunit linker region indicating the three residue insertion (red) in *Hs*cFRS, which when absent may lead to domain swap. **i**, **j** Surface view of the heterotetrameric assembly (αβ)$_{2}$ of both *Pv* and *Hs*cFRS structures (α-subunit is in grey and β-subunit in pink whereas the symmetry related α′’ and β′’ domains are shown in light green and orange respectively). **k** Portion of the sequence alignment showing the linker region between β1 and β2 subdomains of the β-subunit in *Pv*cFRS versus *Hs*cFRS. Source data are provided as a Source Data file.
there are two noticeable, ordered loop conformations in proxi-
inward movement (closed) of residues 507–515 (loop 2, in aux-
iliary pocket) (Fig. 4a–c). Further analysis of the PvcFRS:BRD1389 complex revealed that the diarylacetylene moiety of BRD1389 occupies the L-Phe site, its 4-cyclopropoxy phenyl resides in an auxiliary pocket, and the [6.2.0]-diazabicyclodecane group skirts the ATP site in PvcFRS (Fig. 3d, Supplementary Fig. 7). The PvcFRS:BRD1389 complex is stabilized mainly by hydrophobic and hydrophilic interactions at multiple sites that contribute towards recognition of the diazabicyclodecane core, the 4-cyclopropoxyphenyl and the diarylacetylene appendages (Fig. 3a).

The active site of BRD1389-bound Plasmodium cFRS adopts unique conformations. In this complex, open and close conformational change for residue Arg548 (Fig. 4a, d) is noticeable. In particular, the flexing of Arg548 likely opens the entry point of auxiliary pocket for 4-cyclopropoxyphenyl accommodation, underscoring the induced-fit nature of BRD1389 interaction with PvcFRS (Fig. 4d). In phenyladenylate-bound TtFRS, both the guanidino moiety of Arg321 and Phe216 (PvcFRS; Phe455) provide stacking support to the adenine ring of ATP, whereas in PvcFRS:BRD1389 the corresponding guanidinium (belonging to Arg548) is displaced away from the active site adopting an open conformation (Fig. 4a, d). This can be further investigated in L-Phe-bound HscFRS, where the corresponding Arg463 moves inward (i.e., towards the active site) adopting instead a closed conformation. Additionally, in the PvcFRS:BRD1389 complex there are two noticeable, ordered loop conformations in proximity to BRD1389 binding site within the P1A1 domain: (1) a left-hand outward displacement (open conformation) of residues 443–453 (loop 1, in ATP binding pocket), and (2) a left-hand inward movement (closed) of residues 507–515 (loop 2, in auxiliary pocket) (Fig. 4a–c, Supplementary Fig. 6b, c). In PvcFRS:BRD1389, loop 2 adopts a closed conformation akin to phenyladenylate-bound TtFRS and unlike L-Phe-bound HscFRS (open conformation, Fig. 4c, Supplementary Fig. 6c), whereas loop 1 moves relative to both TtFRS and HscFRS to achieve an open state (Fig. 4b, Supplementary Fig. 6b).

Table 1 BRD1389 potency and selectivity for malaria parasite cFRS over HscFRS.

| Experiment | Organism | Values (nM) |
|------------|----------|-------------|
| EC₅₀       | Pf (3D7) | 13          |
| IC₅₀ (cFRS)| Pf (K1)  | 12          |
|            | Pf       | 12 ± 0.8    |
|            | Pv       | 25 ± 1      |
|            | Pf mutant (L550V) | (1.0 ± 0.05) × 10⁻³ |
|            | Hs       | (12 ± 1.2) × 10⁻³ |
| Kᵣ (cFRS) | Pf       | 4           |
|            | Hs       | 16 × 10⁻⁶  |

Pf: Plasmodium falciparum, Pv: Plasmodium vivax, Hs: Homo sapiens

where it provides an aromatic T-shaped...π interaction at a distance of ~4.2 Å (Fig. 3b). In addition, the diarylacetylene moiety is surrounded by hydrophobic elements of Asn519, Gln457, and Glu459 (Fig. 3b). The urea moiety of BRD1389 is buried in a groove that is composed of Gly506, His508, Glu510, Lys512–513, Leu515, Ile552 and Pro549 (Figs. 3b, e and 4c). The urea/groove interactions are mainly stabilized by hydrophobic contacts, particularly from Pro549 and His508 while residues Val517, Ile483 and Pro458 provide sandwich support to the cyclopropoxy moiety (Figs. 3b and 4c).

The urea component of BRD1389 forms hydrogen bonding interactions with main-chain N-atom of Ser545 (Fig. 3a, b). The above sets of extensive interactions position BRD1389 in an ‘L’ shaped conformation wherein its methoxy methyl group is surrounded by socket residues Arg443, Glu445, His451, and Phe455. Interestingly, the crystallographic pose of BRD1389 is close to the conformation that the inhibitor is predicted to adopt in aqueous solution (Supplementary Fig. 8 and Supplementary Table 2). This similarity highlights the importance of the three-dimensional shape and rigidity of the diazabicyclodecane scaffold in pre-orienting the molecular appendages for optimal target engagement. From overlaying the structure of PvcFRS:BRD1389 with that of phenyladenylate-bound TtFRS, it is apparent that the diazabicyclodecane core and its methoxymethyl extension partially brush past the adenine binding region of the canonical ATP binding site (Figs. 3d and 4b). Given the high binding affinity of BRD1389 for PvcFRS (4 nM, Table 1), it is feasible that BRD1389 blocks the interaction of Plasmodium cFRS with L-Phe first and then with ATP. Indeed, upon incubation of PvcFRS with high concentrations of both BRD1389 and an ATP analogue (the non-hydrolysable adenosine 5'-β,γ-mido) triphosphate, i.e. AMPNP) we observed only binding of BRD1389. This result further supports that BRD1389 binding may occlude ATP engagement. Strikingly, residues in PvcFRS that recognize key ligand components (diazabicyclodecane core, 4-cyclopropoxy phenyl, methoxymethyl and diarylacetylene moieties) are conserved across the apicomplexan phyla, including in human-infecting parasites such as Toxoplasma and Cryptosporidium (Supplementary Fig. 9).

Basis of selectivity and resistance-conferring mutations. Next, to understand the structural basis of selective binding and inhibition by bicyclic azetidines of Plasmodium cFRS versus the human orthologue, we compared atomic structures of PvcFRS: BRD1389 and HscFRS-L-Phe complexes focusing on residues located within 5 Å of the ligand site. Three variant residues Pv-Y458/Hs-1373, Pv-Y480/Hs-F395, Pv-I483/Hs-L398 are located within an auxiliary pocket of PvcFRS (Fig. 5a). This observation indicates that the selectivity of BRD1389 may arise from the terminal cyclopropyl ether (Fig. 5a). Significantly, all protein residues known to confer resistance to bicyclic azetidines upon mutation

Discussion

Through a combination of biochemical and crystallographic studies, we have revealed the molecular underpinning of
Plasmodium cFRS inhibition by bicyclic azetidines. We have shown that BRD1389 inhibits parasite cFRS function by primarily blocking the binding of L-Phe in a competitive manner. The diphenylacetylene moiety of BRD1389 occupies the L-Phe binding site while the [6.2.0]-diazabicyclodecane core partially occludes the ATP binding region. The cyclopropoxyphenyl urea region of BRD1389, in turn, occupies an auxiliary pocket in \( \text{Pv} \) cFRS. Residue variations between the malaria parasite cFRS and the human orthologue in this region underpin the highly selective enzyme inhibition and parasite killing by bicyclic azetidines. Two classes of malaria parasite aaRS inhibitors have been structurally evaluated to date\(^3\). These act either as single-site occupants (cladosporin, an adenosine mimic) or dual site engagers (halofuginone, a mimic of L-Pro and 3' end of tRNA). As BRD1389 occupies both the L-Phe site and an auxiliary pocket within PvcFRS, it represents a novel dual-site malaria parasite aaRS inhibitor (Fig. 6a, 6; right panel).

Overall, our mapping of protein regions and residues that contribute both to cFRS inhibitor selectivity and drug resistance provides a structural platform for designing the next generation of compounds with improved potency and safety profiles. Indeed, the enzyme-inhibitor structure also reveals how the underlying principles of the diversity-oriented synthesis (DOS)\(^{13-15}\) library that yielded this inhibitor scaffold, i.e., inclusion of rigid bicyclic
Fig. 3 BRD1389 occupies the L-Phe site and an auxiliary site on Plasmodium cFRS. a Two dimensional representation (Ligplot) of BRD1389 binding to PvcFRS. BRD1389 is shown as ball-and-stick representation and interaction symbols of residues engaging in hydrophobic interactions with the ligand are highlighted in red. b Close-up view of bound BRD1389 in the active site of α-subunit of PvcFRS. Labelled residues show hydrophobic interactions with BRD1389. c Surface view of the active site and pockets within L-Phe site (purple circle), ATP site (yellow circle) and auxiliary site (black circle). Superimposed structures of L-Phe (purple, PDB 3L4G [https://www.rcsb.org/structure/3l4g]) and phenylalanyl-adenylate (yellow, PDB 2IY5 [https://www.rcsb.org/structure/2IY5]) are depicted. d Close-up view of amino acid and ATP pocket of PvcFRS (cornflower blue) that is superimposed on HscFRS-L-Phe (grey, PDB 3L4G [https://www.rcsb.org/structure/3l4g]). e Close-up view of auxiliary site occupied by BRD1389. Auxiliary site residues are shown which are involved in protein-ligand hydrophobic interactions.

Methods
Prediction of BRD1389 conformation. Calculations were performed using Schrödinger Maestro Version 12.3.012, MMshare Version 4.9.012, Release 2020-1, Platform Windows-x64. Geometry optimization was performed in Jaguar 16 Version 10.7, Release 12, at ultrafine accuracy level, using density functional theory (B3LYP), the 6–31G** basis set, and the Poisson–Boltzmann Finite (PBF) water solvation model. The crystallographic and computed structures were superposed by Max-Imum Common Substructure using the Schrödinger Maestro Superposition tool.

Synthesis of BRD1389. BRD1389 was prepared from known compound (8 R,9 R,10 S,Z)-9-(4-bromophenyl)-6-((4-nitrophenyl) sulfonyl)-10-((trityloxy)methyl)-
Protein expression and purification. Full-length PfFRS was purified according to the earlier published report. Full-length PvFRS was also purified according to the same protocol. In brief, the genes encoding PfFRS alpha subunit (PVX_081300) and beta subunit (PVX_090880) were cloned into E. coli pETM11 and pETM20 plasmids respectively. Both plasmids were co-transformed into E. coli strain B834 and were induced overnight for overexpression with 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) at 16 °C for 18 h. The E. coli cell lysate was first loaded onto a nickel–nitrilotriacetic (Ni–NTA) column (GE Healthcare) and the eluted fraction was further purified with Heparin chromatography (GE Healthcare) to a single band as indicated by SDS–polyacrylamide gel electrophoresis with Coomassie brilliant blue staining. The purified protein was found as a single peak with the elution volume consistent of a homogeneous PfFRS hetero-tetramer on the Superdex 200 analytical gel filtration column (GE Healthcare). The purified PfFRS was concentrated to 25 mg ml⁻¹ and stored at −80 °C in 25 mM HEPES buffer, pH 7.5, 200 mM NaCl, 5 mM βME.

Evaluation of parasite growth inhibition. Plasmodium falciparum strain 3D7 (chloroquine-sensitive) and K1 (chloroquine-resistant) were obtained from Kitasato University and used for testing antimalarial activities in vitro. The cultivation of P. falciparum was conducted according to Trager’s method with some modification. Precisely, parasites were kept in culture flasks with RPMI1640 medium supplemented with 10% human plasma and 2% fresh human erythrocytes and incubated at 37 °C with the gas condition of 5% CO₂ and 5% O₂. The parasitemia (percentage of infected erythrocytes to total erythrocytes) were kept within 0.25–10%. Culture medium was replaced, and fresh erythrocytes were supplied every 2–3 days. Drug susceptibility test was conducted according to Desjardins’s method with some modification. The bicyclic azetidines and known antimalarial agents (chloroquine and artemisinin) were tested at the same time. Precisely, 199.5 µl of parasite cultures (2% hematocrit and 0.75–1% parasitemia) and 0.5 µl of compound solution serially diluted in DMSO were poured into every well in 96-well titer plates and final drug concentrations were set.

Figure 4 BRD1389-bound Plasmodium cFRS adopts unique conformations. a Superimposition of HscFRS (grey; PDB “3L4G [https://www.rcsb.org/structure/3l4g]”) and TfFRS (orange; PDB “2IY5 [https://www.rcsb.org/structure/2IY5]”) structures on PfFRS:BRD1389 complex (cornflower blue). b Opening of Loop 1 (residues 443–453) of PfFRS for BRD1389 binding to accommodate its methoxymethyl group and adoption of a possible open conformation. c Closing of Loop 2 (residues 507–515) in PfFRS:BRD1389 complex. d Open conformation of the Arg548 in PfFRS:BRD1389 complex accommodates its [6.2.0]-diazabicyclodecane core as compared to its orthologue residue in HscFRS (Arg463) and TfFRS (Arg321) which in its closed conformation may clash with this ring.
within 0.001–1 µg ml⁻¹. The plates were kept at 37 °C with the gas condition of 5% CO₂ and 5% O₂ for 72 h and then parasite growth was quantified with Malder’s method to detect plasmodial lactate dehydrogenase with some modification. Precisely, culture plates were kept in freezer overnight and then thawed at 37 °C to disrupt the erythrocytes and parasite cells. In the new 96-well titration plates, 100 µl of enzyme reaction solution (110 mM Li-lactate, 0.5 mM acetylpyridine-adenine dinucleotide, 50 mM Tris (pH 7.5), 10 mM EDTA, 50 mM KCl, and 15 g l⁻¹ PEG6000) and 20 µl of freeze-thaw culture were mixed in each well and then kept at room temperature for 30 min. The detection solution was prepared by mixing equal volume of 2 mg ml⁻¹ nitro blue tetrazolium and 0.1 mg ml⁻¹ phenazine ethosulfate and 20 µl of the solution was added to each well. After the incubation at room temperature in the dark for 90 min, absorbance at 660 nm was analyzed and IC₅₀ values were calculated from dose response curve.

**Enzyme inhibition assays.** These were done using malachite green assay as per earlier published reports. Briefly, the reaction was performed for 100 µM ATP, 50 µM L-phenylalanine and 100 nM recombinant PhRS enzymes (Pf, Pv, Hs, Py mutant (L550V)) in a buffer containing 30 mM HEPES (pH 7.5), 150 mM NaCl, 30 mM KCl 50 mM, MgCl₂, 1 mM DTT, and 2 U/ml E. coli inorganic pyrophosphatase (NEB) at 37 °C. Enzymatic reactions (50 µl total volume) were performed in clear, flat-bottomed, 96-well plates (Costar 96-well standard microplates). The reaction mixture was incubated for 2 h at 37 °C. The reaction was stopped by adding 12.5 µl of malachite green solution to the reaction mixture and levels of inorganic phosphate (Pi) were detected after incubation of 5 min at room temperature. Absorbance was the measured at 620 nm using a Spectramax M2 (Molecular Devices). Reactions without FRS enzyme were performed as background controls, values of which were subtracted from the reactions with enzyme. BRD1389 was added to the aminoclay assay reaction buffer in varying concentrations ranging from 0.1 nM to 10,000 nM. The IC₅₀ value for the data is shown for three replicates as the mean ± SD.

**Surface plasmon resonance experiments.** These were carried out on a Biacore T200 instrument (GE Healthcare) at 25 °C. The binding experiments were performed in buffer 10 mM phosphate buffered saline (PBS), pH 7.4, containing 5% dimethyl sulfoxide (DMSO). The flow system was primed with the running buffer before the initiation of the experiment. Both PfFRS and HscFRS were immobilized to Sensor Chip CM5 by standard amine coupling chemistry using N-hydroxysuccinimide (NHS) and ethyl(dimethylaminopropyl) carbodiimide (EDC) to an immobilization level of approximately 1500 RU. The binding experiments were carried out in a single cycle kinetics mode. BRD1389 was serially diluted in running buffer and injected at a flow rate of 50 µl min⁻¹ across both surfaces for 60 s and dissociations were set up for 120 s. The data analysis was done using Biacore evaluation T200 Evaluation software (GE Healthcare) and after applying the solvent correction, the data was fitted into the 1:1 binding evaluation method to determine the equilibrium dissociation constants (Kd).

**PfFRS mode of inhibition studies.** To establish the mode of inhibition of BRD1389, data sets (generated using the malachite Green assay platform as described in the above enzyme inhibition assays method section) were collected by varying both the inhibitor and substrate concentrations. Using Graph Pad Prism each data set was individually fitted to the Michaelis–Menten equation and the resulting Lineweaver-Burk plots were examined for diagnostic patterns of
competitive, mixed or un inhibited. Data sets were then globally fitted to the appropriate model (with Eqs. 1 and 2 used for competitive and mixed inhibition respectively).

\[
V = \frac{V_{\text{max}}[S]}{K_m (1 + \frac{[S]}{[S]})}
\]  

(1)

\[
V = \frac{V_{\text{max}}[S]}{K_m (1 + \frac{[S]}{[S]}) + K_m (1 + \frac{[S]}{[S]})}
\]  

(2)

Crystallographic, data collection and structure determination. The purified PfFRS and PcFRS proteins were used for crystalization by the hanging-drop vapour-diffusion method at 293 K using commercially available crystalization screens (Index, JCSG-plus, Morpheus, PACT premier, PGA, Crystal Screen, PEG/ION and ProlFlex Hampton Research and Molecular Dimensions). Initial screening was performed in 96-well plates using a nano drop dispensing mosquito robot (TTP Labtech). Three different drop ratios were used for the crystalization trials by mixing 75, 100, or 50 nl purified protein solution with 75, 50, or 100 nl reservoir solution, respectively (i.e., 1:1, 2:1, and 1:2 drop ratios). Each of the drops was equilibrated against 100 ml of the corresponding reservoir solution. Before crystalization, PfFRS was diluted to 12 mg ml⁻¹ with 5 mM BRD1398, 5 mM MgCl₂, and 4 mM JFME, and then incu bated for 30 min. Diffraction quality PfFRS-BRD1398 crystals were obtained in PGA screen F4 [0.1 M sodium cacodylate (pH 6.5), 3% w/v poly-g-glutamic acid (Na₅ form, low molecular weight), 3% w/v PEG20000, 0.1 M ammonium sulphate, 0.3 M sodium formate]. The crystals were mounted in nylon loops (Hampton Research) or lioth loops (Molecular Dimensions) after being soaked for 10-30 s in a cryoprotectant containing the corresponding crystalization mother liquor with 20%(v/v) glycerol. The crystals were subsequently flash-cooled in liquid nitrogen. X-ray diffraction data were collected on beamline I24 at Diamond Light Source (DLS), United Kingdom at a wavelength of 0.9888 Å. The data were processed by the xia2 auto-processing pipeline 20 using DIALS21 for integration. The initial model for PfFRS-BRD1398 was determined by the molecular-replacement (MR) method using Phaser22 with HsFRS (PDB entry 3L4G_OP [https://www.rcsb.org/structure/3l4g]) as the template. It was then subjected to AutoBuild23 that provided a partial model with Rwork/Rfree of 31/41% for ∼800 residues in 29 fragments. Subsequently, the model was manually built and completed by iterative cycles of building using COOT24 and refinement using Refmac25. After each cycle of manual building and refinement, the models were inspected and manually adjusted to correspond to the 2Fₐ-Fᵄ and Fᵄ-Fᵂ electron density maps. During refinement, the ligands BRD1398 and Mg²⁺ ion were added based on positive peaks in difference Fourier maps and the model was subjected simulated annealing refinement using phenix.refine in Phenix26. The final model was refined to 3.0 Å resolution with Rwork/Rfree of 21.4/ 28.8%. The stereo-chemical quality of the model was analysed using MolProbity27 and the model has good geometry quality and all residues are in favoured/allowed (92.8%) regions of the Ramachandran plot. We additionally carried out crystal packing analysis of PfFRS-BRD1398 complex using COOT24. Statistics of data collection and structure refinement are given in Supplementary Table 1. The atomic coordinates and structural factors have been deposited into Protein Data Bank with accession code 7BY6 [https://www.rcsb.org/structure/7BY6].

Kinetic Parameter Determination. Michaelis constant, Km for the ATP and L-Phe substrates for PfFRS wild type (WT) and PfFRS mutant (L550V) was determined using the malachite green assay 6 in a buffer containing 30 mM HEPES (pH 7.5), 150 mM NaCl, 30 mM KCl, 50 mM MgCl₂, 1 mM DTT and 2 U/ml E. coli inorganic pyrophosphatase (NPP). ATP Km was determined using saturating concentration of L-Phe (1000 μM) and varying the concentration of ATP from 1000 to 0.01 μM. Similarly, the Km for L-Phe was determined using saturating concentration of ATP (500 μM) and varying the concentration of L-Phe from 1000 to 0.0001 μM. In both cases the reaction was carried out for 2 h at 37 °C. Data were fitted to Michaelis-Menten equations using Prism graph 6.0 software.

Sequence analysis and structure presentation. Protein sequences were aligned using the program Cluster Omega28 by using the default settings. All structural superimpositions and preparation of figures was conducted using Chimera29 and PyMol30.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The authors confirm that all relevant bioinformatic and synthesis data supporting the findings are included in the paper and its supplementary file. Structural data have been deposited into Protein Data Bank with accession code 7BY6 [https://www.rcsb.org/structure/7BY6]. Public datasets ("3L4G [https://www.rcsb.org/structure/3l4g]", "2IYS [https://www.rcsb.org/structure/2IYS") were used in this study. Source data for figures Table 1, Figs 1b–d, 5c, d and Supplementary Figs. 3b, 4b are provided with this paper.
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Author contributions
A.S. designed and managed the study. M.S., N.M., M.Y., and A.S. purified the protein, crystallized, and solved the structure. M.S. performed all biochemical assays. K.H. helped with data collection and S.P. helped with biochemical analysis. B.Me., E.C., A.G., B.Mi., F. G.F., and S.L.S. designed and synthesized BRD1389. We wrote the manuscript.

Competing interests
S.L.S. is a shareholder and serves on the Board of Directors of Jnana Therapeutics; is a shareholder of Forma Therapeutics and Decibel Therapeutics; is a shareholder and advises Kojin Therapeutics, Kisbee Therapeutics and Eikonizo Therapeutics; serves on the Scientific Advisory Boards of Eisai Co., Ltd., Ono Pharma Foundation, Exo Therapeutics, Biogen, Inc. and F-Prime Capital Partners; and the Board of Advisers of the Genomics Institute of the Novartis Research Foundation; and is a Novartis Faculty Scholar. The Broad Institute and Harvard have filed patent applications relating to work described in this manuscript, including “Compounds and Methods for Treating Parasitic Diseases” (PCT/US18/23270, filed on March 20, 2018 and published as WO18/175385), and “Apicomplexan Parasite Inhibition” (unpublished).

Additional information
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