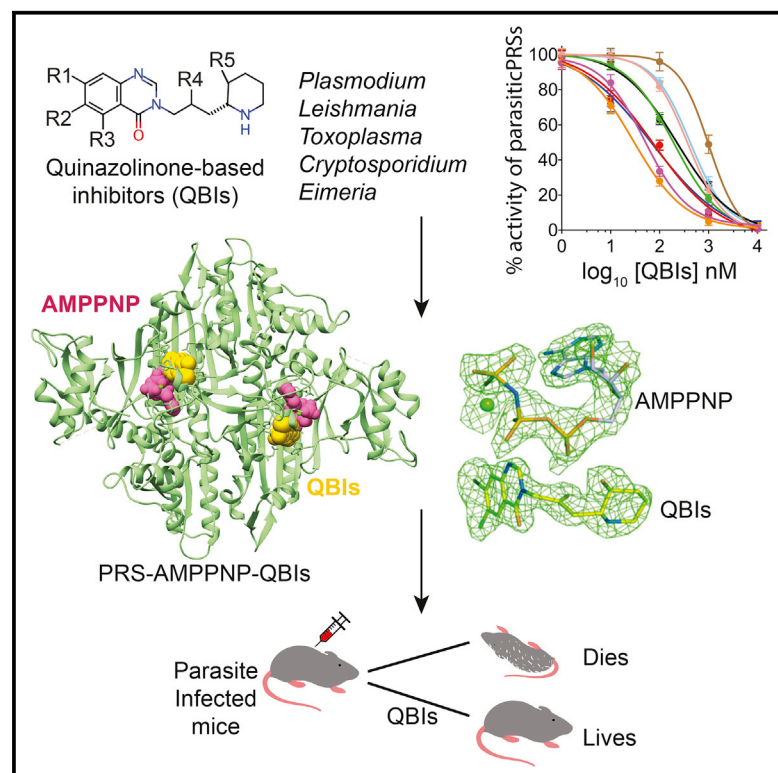


Structure

Targeting Prolyl-tRNA Synthetase to Accelerate Drug Discovery against Malaria, Leishmaniasis, Toxoplasmosis, Cryptosporidiosis, and Coccidiosis

Graphical Abstract



Authors

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In Brief

Synthesis and enzyme inhibition in multiple parasites, described by Jain et al., provides a new facet to quinazolinone-based inhibitors (QBIs). Multi-parasite drugging studies, coupled with structures of protein-drug complexes, reveal structural basis of QBI action. Structure-guided optimization and mice model studies reveal a top hit compound.

Highlights

- Quinazolinone-based (QBIs) selective inhibition of prolyl-tRNA synthetase (PRS)
- QBIs inhibit *Plasmodium*, *Leishmania*, *Toxoplasma* and *Cryptosporidium* growth
- High-resolution PRS-QBI structures reveal active site plasticity
- Inhibitor 5 (In-5) displays good drug-like properties and clears rodent malaria

Accession Numbers

5XIF
5XIG
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5XII
5XIJ
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Targeting Prolyl-tRNA Synthetase to Accelerate Drug Discovery against Malaria, Leishmaniasis, Toxoplasmosis, Cryptosporidiosis, and Coccidiosis

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<http://dx.doi.org/10.1016/j.str.2017.07.015>

SUMMARY

Developing anti-parasitic lead compounds that act on key vulnerabilities are necessary for new anti-infectives. Malaria, leishmaniasis, toxoplasmosis, cryptosporidiosis and coccidiosis together kill >500,000 humans annually. Their causative parasites *Plasmodium*, *Leishmania*, *Toxoplasma*, *Cryptosporidium* and *Eimeria* display high conservation in many housekeeping genes, suggesting that these parasites can be attacked by targeting invariant essential proteins. Here, we describe selective and potent inhibition of prolyl-tRNA synthetases (PRSs) from the above parasites using a series of quinazolinone-scaffold compounds. Our PRS-drug co-crystal structures reveal remarkable active site plasticity that accommodates diversely substituted compounds, an enzymatic feature that can be leveraged for refining drug-like properties of quinazolinones on a per parasite basis. A compound we termed In-5 exhibited a unique double conformation, enhanced drug-like properties, and cleared malaria in mice. It thus represents a new lead for optimization. Collectively, our data offer insights into the structure-guided optimization of quinazolinone-based compounds for drug development against multiple human eukaryotic pathogens.

INTRODUCTION

Protozoan classes of aconoidasida and kinetoplastida include members of the genera *Plasmodia*, *Toxoplasma*, *Leishmania*, *Cryptosporidium* and *Eimeria*. These unicellular organisms infect and cause severe parasitic diseases in ~600 million humans and cause >500,000 deaths yearly (WHO, 2015). Of these, *Plasmodia* and *Leishmania* require a vector and a human host to complete their life cycles, while *Toxoplasma*, *Cryptosporidium* and *Eimeria* infect via the oral route (Chapman et al., 2013; Greif et al., 2001; Mears et al., 2015; Neville et al., 2015; Tzipori and Ward, 2002). The emergence and quick spread of drug-resistant parasites further highlights the need to exploit vulnerabilities in these parasites to ultimately eradicate or control their infections (Dondorp et al., 2009; Tilley et al., 2016). Thus, the discovery of new chemical classes of anti-parasitic compounds and new druggable targets is essential. Several recent studies have highlighted the utility of targeting parasite enzymes involved in protein synthesis (Bhatt et al., 2009, 2011; Herman et al., 2015; Hoepfner et al., 2012; Hussain et al., 2015; Istvan et al., 2011; Jackson et al., 2011; Jain et al., 2014, 2015; Kato et al., 2016; Khan et al., 2011, 2013a, 2013b, 2014; Koh et al., 2012; Palencia et al., 2016; Sonoiki et al., 2016). Aminoacyl-tRNA synthetases (aaRSs) couple specific tRNAs with selected amino acids for ribosome-based protein synthesis; these are housekeeping enzymes with essential roles in proteostasis. A growing number of reports suggest aaRSs as valuable targets in pathogenic organisms (Herman et al., 2015; Hoen et al., 2013; Hoepfner et al., 2012; Hussain et al., 2015; Jain et al., 2015; Kato et al., 2016; Khan et al., 2013a; Koh et al., 2012; Palencia et al.,

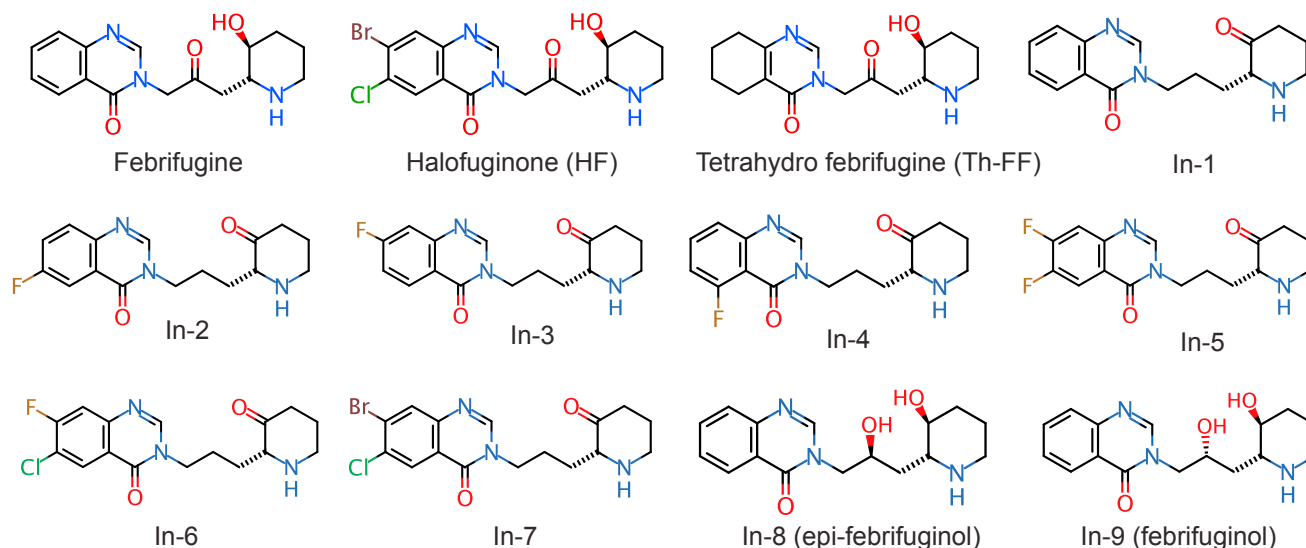


Figure 1. 2D Structures

The chemical structures of synthesized compounds that form the QBI series.

2016; Sonoiki et al., 2016). Others, and ourselves, have shown the remarkable ability of halofuginone (HF) to target the *Plasmodium falciparum* prolyl-tRNA synthetase (PfPRS) enzyme (Herman et al., 2015; Jain et al., 2014, 2015). HF is a derivative of the natural quinazolinone-type alkaloid febrifugine that comes from the *Dichroa febrifuga* herb (Ryley and Betts, 1973). Another example of targeting orthologous proteins in related parasites has yielded the proteasome as an excellent conserved drug target against kinetoplastid parasites (Jain and Sharma, 2017; Jain et al., 2017; Khare et al., 2016).

Here we show that several human parasite PRS enzymes from both apicomplexan and kinetoplastid lineages are exceptionally druggable. Our quinazolinone-based, structure-guided derivatives show striking *in vitro* inhibition of multiple parasitic PRSs, and are both potent and selective against the *Plasmodium* parasite *in vivo* in mice models. Using extensive structural data, we have identified an inhibitor (In-5) as a top lead compound that clears mouse malaria parasite *Plasmodium berghei* in an animal infection model. The PRS-drug co-crystal structures reveal active site plasticity that accommodates diversely substituted compounds, an enzymatic feature of PRSs that can be leveraged for developing drug-like properties of quinazolinones for multiple parasites. This work hence lays a foundation for tailoring quinazolinone-based derivatives for the development of leads against parasites that cause malaria, leishmaniasis, toxoplasmosis, cryptosporidiosis and coccidiosis.

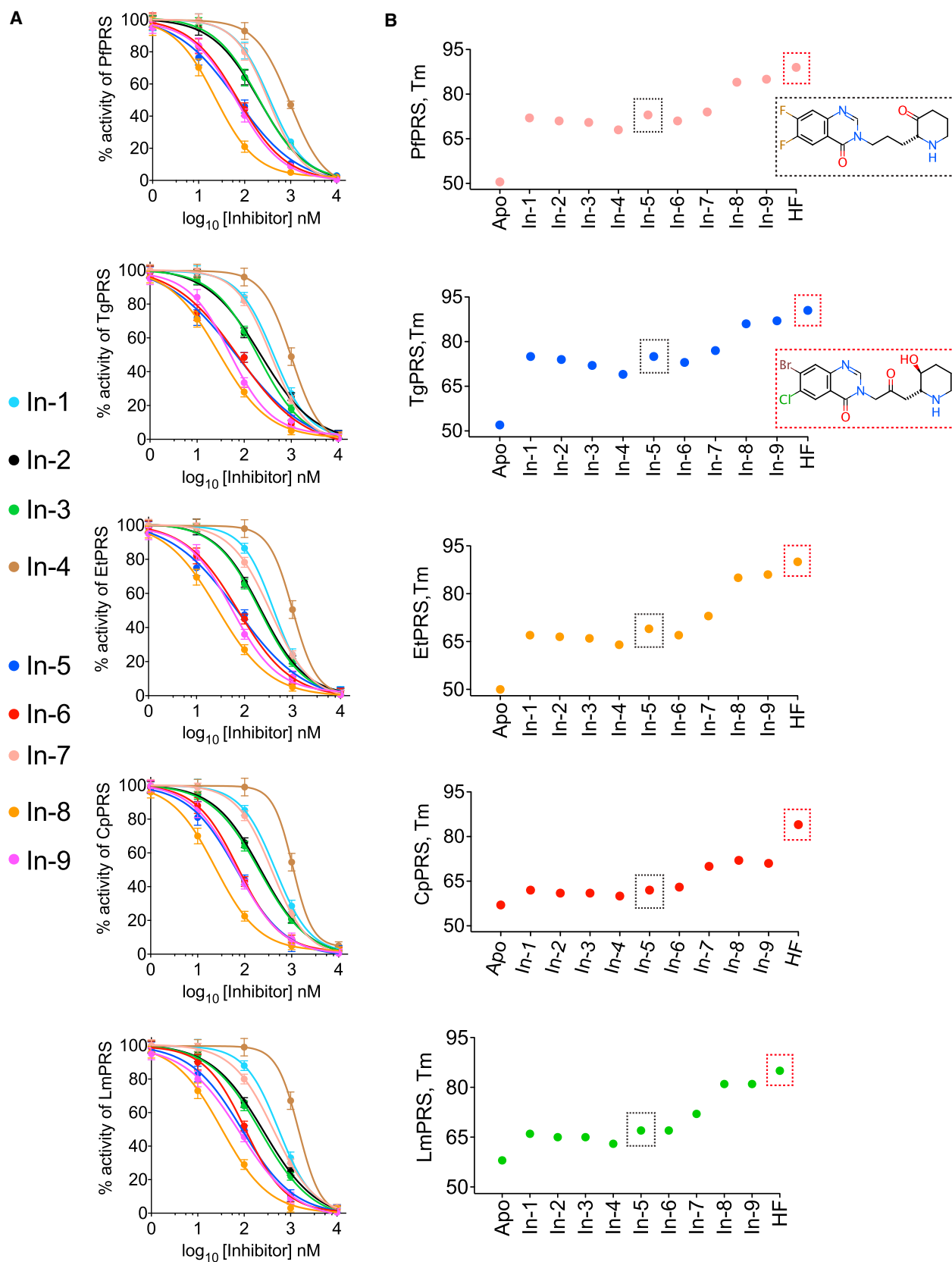
RESULTS

Synthesis of Quinazolinone Derivatives and Target Validation

Taking cues from the high-resolution structures of HF complexed with PfPRS-HF and *Homo sapiens* prolyl-tRNA synthetase (HsPRS-HF), we synthesized a new series of nine compounds centered on the quinazolinone scaffold with substitutions around the linker and the quinazolinone ring (Figure 1).

Critical modifications involved replacement of the linker keto group with a hydroxyl group or in another compound its complete removal (Figure 1). We selected this position to weaken the non-specific interactions between off-target protein atoms and the piperidine keto group based on the chemical knowledge of Michael-acceptor formation and the atomic structure of the PfPRS-HF complex. We estimated that, in the absence of this interaction compounds would still interact but in a more target-specific manner (Zhu et al., 2009). Along with this, the hydroxyl group at the piperidine ring was replaced with a keto group (a double donor), and these new compounds also differed in the halogen positioning on the quinazolinone moiety (Figure 1). These structure-guided quinazolinone derivatizations provided us with a novel series of compounds, and hereafter we refer to this chemical cohort as the QBI series (quinazolinone-based inhibitors). Details of the synthesis and purification of QBIs are given in the STAR Methods section.

To investigate the target specificity of these QBIs, we performed aminoacylation assays with recombinantly produced, purified PRSs from five parasites: *P. falciparum* (PfPRS), *Toxoplasma gondii* (TgPRS), *Leishmania major* (LmPRS), *Eimeria tenella* (EtPRS), and *Cryptosporidium parvum* (CpPRS) (Table S1). We selected the non-*Plasmodium* parasites due to our observation that HF binding residues in these pathogen PRSs are essentially identical, thus offering us an experimental window to probe their active sites using quinazolinone derivatives (Figure 1). In these aminoacylation assays, QBI concentration ranges from 0.1 to 10,000 nM were used (Figure 2A). For PfPRS, our data indicated the half maximal inhibitory concentration (IC₅₀) values of 350, 210, 205, 980, 68, 72, 330, 23 and 67 nM respectively, for QBIs named from In-1 to In-9 (Figure 2A; Table 1). Similarly, IC₅₀ values for other four parasitic PRSs and HsPRS were measured and analyzed (Table S2; Figure 2A). Experimental data suggested that IC₅₀ values ranged from 30 nM to 1.5 μ M for the parasitic PRSs and from 42 nM to 2 μ M for HsPRS. These screening experiments revealed that highest potency inhibition,



(legend on next page)

Table 1. QBI Compounds Tested in *P. falciparum* Culture with the Purified PfPRS Enzyme along with the Selectivity Index against Mouse L929 Fibroblast Cells and Human HeLa Cells

Serial No.	Enzyme Inhibition IC ₅₀ (nM)	Parasite Inhibition EC ₅₀ (nM)	Selectivity Index ^{a,b}
In-1	350 ± 140	110 ± 35	1,015, 1,020
In-2	210 ± 80	60 ± 20	700, 750
In-3	205 ± 75	50 ± 18	170, 158
In-4	980 ± 310	350 ± 120	800, 810
In-5	68 ± 35	20 ± 9	915, 915
In-6	72 ± 28	25 ± 11	710, 900
In-7	330 ± 130	100 ± 38	690, 675
In-8	23 ± 8	5 ± 2	390, 401
In-9	67 ± 23	18 ± 6	120, 115

^aTested in mouse L929 fibroblasts cells.

^bTested in HeLa cells.

across all parasitic PRSs, was achieved by In-5 (containing two F atoms) and In-6 (F and Cl) which differed in the halogenation on the quinazolinone moiety (Figure 1). We then investigated the ATP-assisted binding of QBIs with parasitic PRSs and HsPRS using thermal shift assays (Figure 2B). AMPPNP/ANP (non-hydrolysable ATP analog) binding to PRSs indeed enhanced binding interactions with these QBIs (Figure 2B). However, the degree of binding observed for HsPRS was significantly lower when compared with five parasitic PRSs (Figures 2B and S1), suggesting that subtle substitutions in the parent HF moiety could lead to enhanced selectivity for pathogen PRSs over the human counterpart. Based on specificity for parasitic active sites, the bihalogenated compounds In-5 and In-6 again seemed most promising. Nonetheless, we tested all nine compounds in cell-based parasite growth inhibition assays (Figure 3). For each inhibitor, dose-dependent growth inhibition assays were performed to calculate the half maximal effective concentration (EC₅₀) values, which ranged from 5 nM for In-8 to 350 nM for In-4 in the case of malaria parasites (Figure 1; Table 1). Cytotoxicity profiling in mouse fibroblasts L929 cells and human HeLa cells revealed that selectivity indexes (ratio of the EC₅₀ in mouse or human cells to EC₅₀ in the parasites) were more than 500 for most QBIs, and peaked to ~900 for In-5 and ~1,000 for In-1 (Table 1). For other parasites, the best EC₅₀ values were: ~275 nM for HF against *C. parvum*, ~1.5 nM, ~900 nM, ~9 μM, and ~2 μM for HF, ThFF, In-1 and In-9 respectively in the case of *T. gondii* and ~3 nM for HF against intracellular amastigote stage growth of *L. major* (Figure 3, Table S3). Based on comparative growth inhibition data from *Cryptosporidium*, *Toxoplasma*, *Leishmania* and *Plasmodium*, we inferred that HF was effective at nanomolar concentrations for these four (we were unable to do this for *Eimeria*). From our QBI series, In-1 and In-5 showed the best potency against *Plasmodium*. The probable cause for the stark dif-

ferences in the potencies of QBIs in these four parasites, despite nanomolar range target IC₅₀ values and binding affinity, can potentially be attributed to the differential permeability of these compounds. The QBI series evidently traverses through the red blood cell membrane in the case of *Plasmodium* parasites; however, most likely not sufficiently well through epithelial cells for *Cryptosporidium* parasites, fibroblast cells for the *Toxoplasma* parasites, and macrophage cells for *Leishmania* parasites. This differential permeability due to different halogen atoms might also explain the varied EC₅₀ values among these nine compounds which all have a common and conserved protein target. Our data confirm the enhanced potential of these QBIs as anti-malarials, and validate strongly PRSs as remarkably attractive drug targets in these pathogens.

Atomic Interactions of Parasitic PRSs with QBIs

Phylogenetic analyses of pathogen and human PRSs indicated that these enzymes were broadly of two types, despite their host organism classification into three taxonomic classes of aconoidasida, kinetoplastida and mammalia (Figure 4A). Subtype I consists of PRSs from *Cryptosporidium*, *Leishmania* and *H. sapiens*, while II are contributed from *Toxoplasma*, *Eimeria* and *Plasmodium*. The PRS sequences in all six of these organisms are mostly conserved, with a few differences in the residues that are distal to the catalytic sites (Figure S2). To investigate the atomic basis for differences in subtypes I and II, and in their interactions with HF, we resolved crystal structures of pathogen PRSs in apo and/or drug-bound forms. Investigation of subtype I PRSs such as CpPRS-HF-ANP, LmPRS-apo and HsPRS-HF-ANP with subtype II, EtPRS-HF-ANP, TgPRS-apo and TgPRS-HF-ANP revealed essentially identical interactions between HF and PRSs with differences in a small disordered loop (333–337 in PfPRS) close to the active site (Figures 4A, S2, and S3; Movie S1), and in their metal ion (Mg²⁺ and Zn²⁺) requirements. The flexibility of this loop is depicted in Figure S4. The EtPRS has a single octahedral coordinated Mg²⁺ ion in the active site to chelate ATP phosphates (β and γ) (Figure 4B). However, TgPRS requires two Mg²⁺ ions while CpPRS lacks Mg²⁺ ion in the active site. Strikingly, only subtype I PRSs had a Zn²⁺ ion complexed via tetrahedral coordination in the PRS active site using either three (in LmPRS) or four (in HsPRS and CpPRS) closely placed cysteine residues. In subtype II, these cysteines are replaced with either serine or threonine, thus making subtype II seemingly incapable of zinc coordination (Figure 4C).

We next undertook to dissect the structural basis for the differential binding/activity of QBI-PRS interactions. For this we developed the TgPRS system for co-crystallization and structural elucidations as it was the most amenable enzyme from the above PRS cohort for studying the atomic resolution details of QBI-PRS contacts (Tables S4 and S5). We generated a series of drug-AMPPNP complexes with TgPRS using In-1, In-5, In-6, In-9 and ThFF (Figures 5 and S5). We observed remarkable

Figure 2. In Vitro Assessment

(A) Aminoacylation activity inhibition assays were performed for five parasitic PRSs namely PfPRS, TgPRS, EtPRS, CpPRS, and LmPRS with nine QBI compounds. The dose-dependent sigmoidal curves were used to calculate IC₅₀ values for each compound for the respective parasite PRS. Error bars represents means ± SD.

(B) Thermal shift assays to evaluate the melting temperature-based binding affinity for each QBI with pathogen PRS. Most QBIs showed similar trends based on the melting temperature shifts. The inset red and black dotted box shows the 2D structures of HF and In-5.

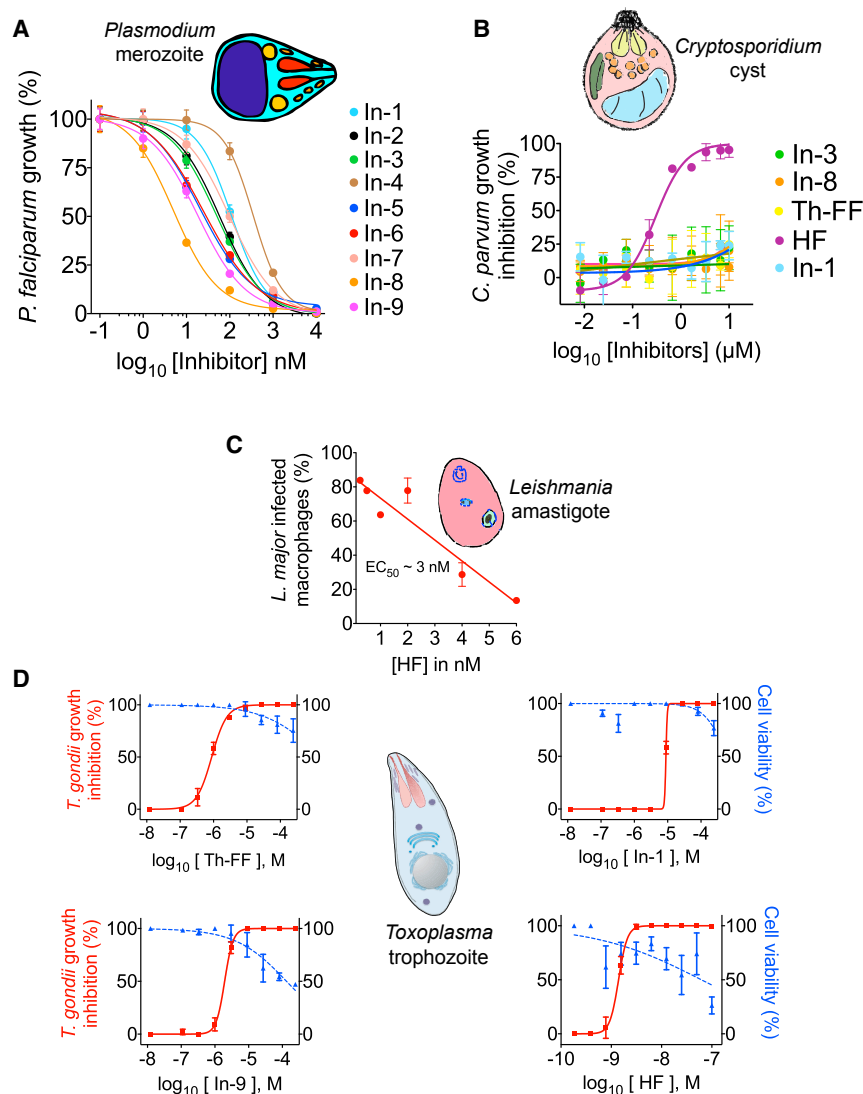


Figure 3. Cellular Parasitic Growth Inhibition

(A) Blood stage *Plasmodium falciparum* growth inhibition assays using nine QBI compounds. The plots between percent growth versus different concentrations of QBI showed a sigmoidal curve and yielded EC_{50} values for each QBI compound that ranged between 5 and 350 nM.

(B) *Cryptosporidium parvum* growth inhibition assays using In-1, In-3, In-8, ThFF, and HF. The sigmoidal curve for HF provided an EC_{50} value of 270 nM and >10 μ M for the rest.

(C) *Leishmania major* amastigote growth inhibition assay using HF yielded an EC_{50} value of 3 nM.

(D) *Toxoplasma gondii* growth inhibition assay using In-1, In-9, ThFF and HF yielded the EC_{50} values of 8.9 μ M, 1.9 μ M, 0.88 μ M and 1.4 nM respectively. Error bars represents means $\pm$ SD.

to the removal of the Michael addition-prone keto group at the linker that may have caused off-target effects in host cells (Figure 5B) (Herman et al., 2015; Zhu et al., 2009).

Particularly noteworthy is the observation that in TgPRS complexes with In-5 and/or In-6, the linker chain and the piperidine ring of both In-5 and In-6 take alternate double conformations and interact with the active site residues differently (Figure 5B). Whether these alternate conformational states of the piperidine ring allow these two inhibitors to perform better in terms of selectivity over the mammalian cells, with selectivity index (SI) values of ~ 900 and ~ 700 for In-5 ($IC_{50} \sim 68$ nM) and In-6 ($IC_{50} \sim 72$ nM) remains to be determined. The alternate piperidine ring conformations in In-5 and In-6 are likely driven by the evident steric

hindrances between phosphates and the carbonyl group of these compounds, and possibly due to the two halogen atoms at carbon positions 6 and 7 on the quinazolinone rings (Figure 1).

plasticity in the TgPRS active site to accommodate each of these derivatives (Figure 5). There are no major differences between seven structures of TgPRS complexes (root-mean-square deviation of 0.40 Å). We found two Mg^{2+} ions displaying octahedral coordinations with AMPPNP phosphates and with water molecules in the active sites of all TgPRS-drug complexes (Figure 5A). Well-defined electron density for all QBIs was found in the active sites of both subunits of TgPRSs (Figures 5 and S6). The L-Pro binding pockets of TgPRS in the two subunits are essentially identical, as is the case for AMPPNP binding interactions. The key residues that participate in direct interactions with AMPPNP and inhibitor molecules are as follows: Glu441, Arg470, Glu472, Arg481, His560 and Thr592 (Figure 5). Glu441 and His560 form a H-bond with the amine group of the hydroxyl-piperidine ring of these inhibitors. Arg470 interacts with AMPPNP phosphate (α - and β -phosphates) and with the linker oxygen atom. Arg481 and Glu472 stabilize β - and γ -phosphates of AMPPNP, while Thr592 stabilizes the adenine ring of AMPPNP (Figures 5 and S5). The observed high selectivity of QBIs and, in particular of In-5, in the context of *Plasmodium* parasite assays is likely due

hindrances between phosphates and the carbonyl group of these compounds, and possibly due to the two halogen atoms at carbon positions 6 and 7 on the quinazolinone rings (Figure 1).

In Vivo Validation of the Top QBIs

Encouraged by our target- and cell-based validations, and with access to our data on structural underpinnings of TgPRS-drug complexes, we sought an *in vivo* system to test the efficacy of QBIs. To assess these, Peters' suppressive test (Peters, 1975) in a *P. berghei* mouse model of malaria was conducted. All nine QBIs were dosed daily at 25 mg/kg orally, and this treatment reduced *P. berghei* parasite burden by $>99\%$ by day 4 relative to control untreated mice (with varying degrees of efficacy within QBIs, see Figure 6A; Table S6). The derivatives In-2, In-5 and In-6 were best in terms of therapeutic indexes of ~ 97 , ~ 68 and ~ 77 respectively (Figure 6A and Table S6). Compound In-5 acted in the *in vitro* experiments with IC_{50} and EC_{50} values of 68 and 20 nM respectively, and an SI of ~ 900 (ratio of EC_{50} for mammalian cells to EC_{50} for parasite cells). Other inhibitors like febrifugine, In-1, In-8 and In-9 performed poorly with therapeutic

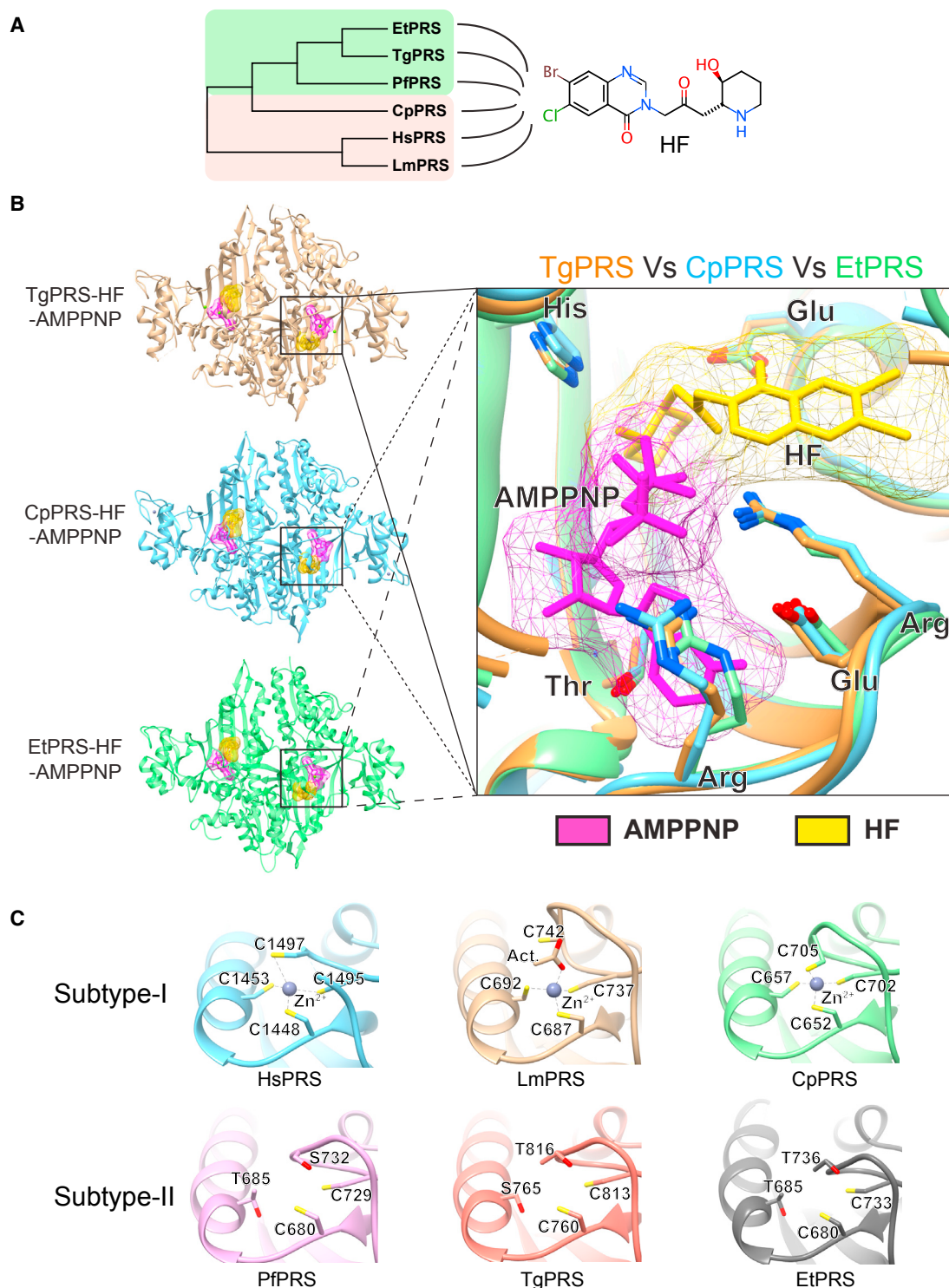


Figure 4. HF in Complexation with Parasitic PRSs

(A) Five parasite and the human PRSs classified in two subtypes. Subtype I is comprised of *Homo sapiens* PRS (HsPRS), *Cryptosporidium* (CpPRS) and *Leishmania* (LmPRS), while the subtype II has *Plasmodium* (PfPRS), *Toxoplasma* (TgPRS) and *Eimeria* (EtPRS). All six PRSs are inhibited by HF.

(B) The superpositions of X-ray structures of three parasitic PRSs in ternary complex with HF and AMPPNP are shown here as ribbon diagrams: tan for TgPRS, blue for CpPRS and green for EtPRS. Critical PRS residues are depicted here with three letter codes and show sequence conservation along with identical residual side-chain rotamers. The molecular mesh surface of HF is in yellow and AMPPNP is in magenta, showing AMPPNP-assisted HF fitting into the active sites.

(C) Cropped-in PRSs view to depict differences in six zinc binding domains for the two subtypes along with the coordination of the zinc atom in subtype I PRSs. Please see [Movie S1](#).

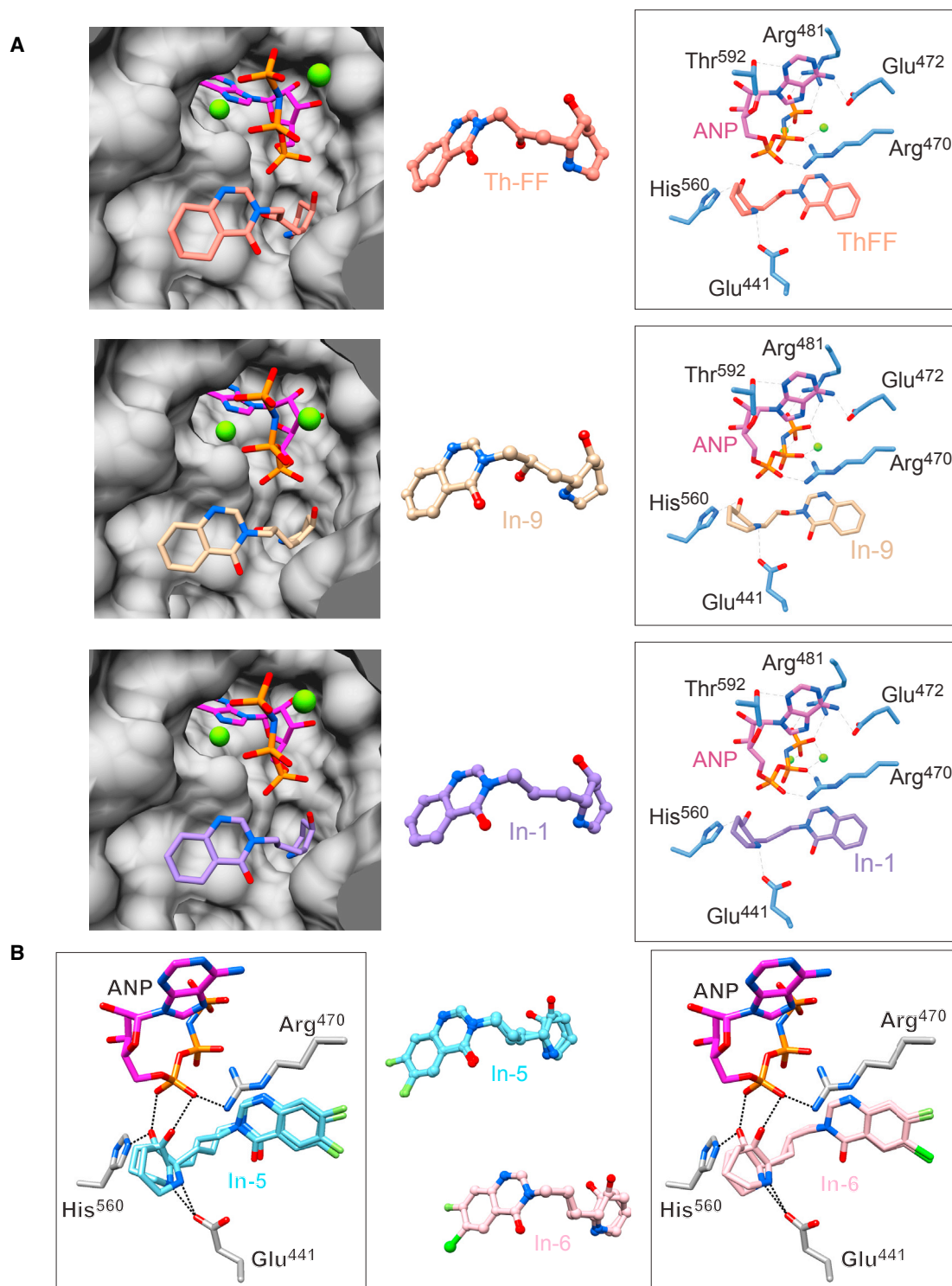


Figure 5. QBIs in Complexation with *Toxoplasma gondii* PRS

(A) Three co-crystal structures of ternary complexes of TgPRS-ThFF-AMPPNP, TgPRS-In-9-AMPPNP and TgPRS-In-1-AMPPNP showing surface views of the active sites and drug interactions.

(B) Ternary complexes of TgPRS-In-5-AMPPNP and TgPRS-In-6-AMPPNP that show alternate conformations of the piperidine ring of In-5 and In-6 in the active sites. The piperidine ring of these two compounds display dual conformations which results in alternative interactions with TgPRS residues Arg470, Glu441 and His560.

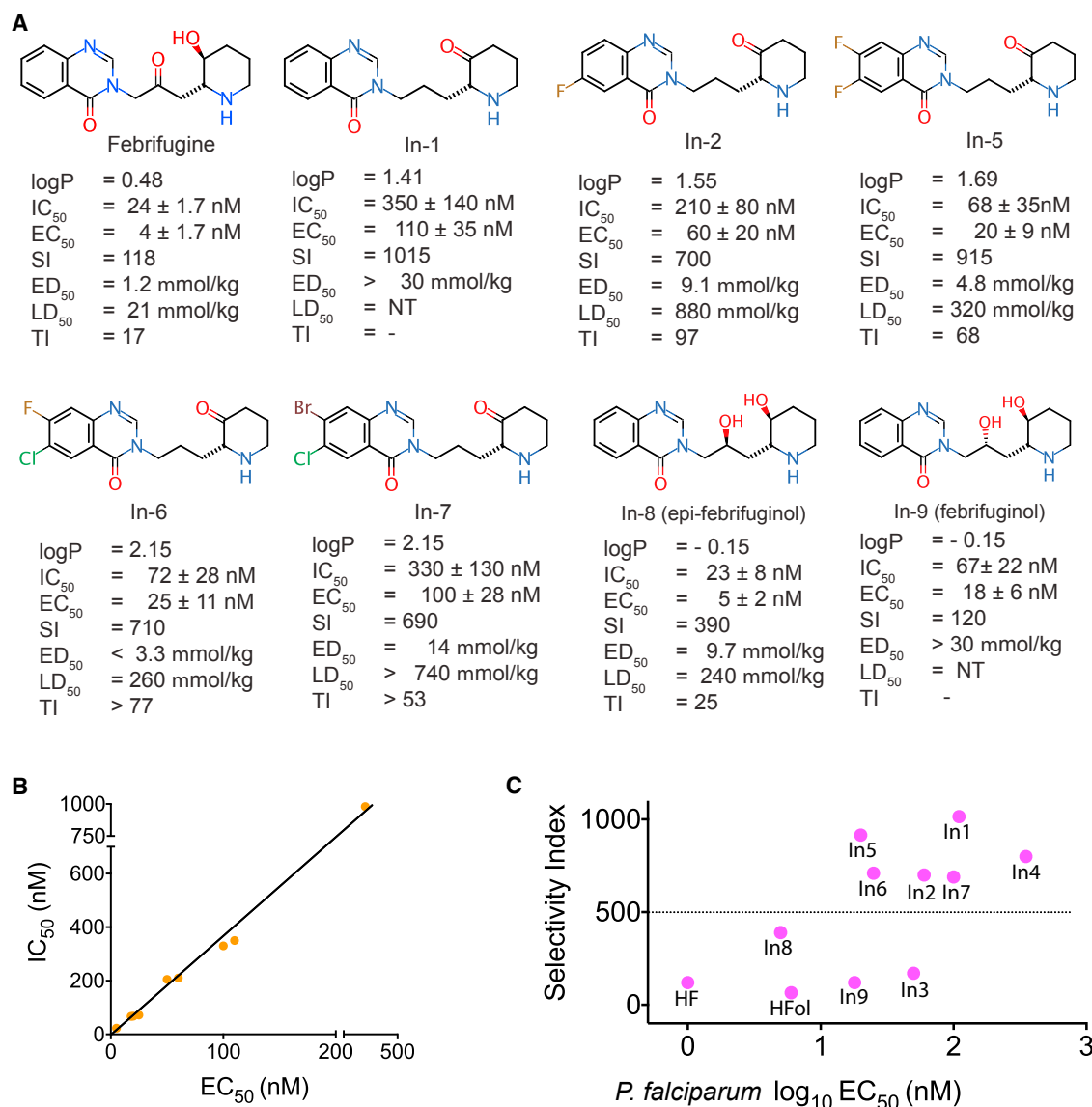


Figure 6. In Vivo Mouse Malaria Studies

(A) Summary of drug characteristic values obtained for each of the newly synthesized QBIs tested against malaria parasite in mouse models (ED₅₀, dose required to achieve 50% of the desired response in 50% of the population; LD₅₀, dose required to achieve 50% mortality from toxicity of the compound; TI, therapeutic index is calculated by the LD₅₀ value divided by the ED₅₀ value). Along with this, logP, EC₅₀, IC₅₀, and SI values are shown for comparison (logP, partition coefficient of a molecule between an aqueous and lipophilic phases; IC₅₀, inhibitor concentration where the enzyme activity is reduced by half; EC₅₀, inhibitor concentration where the parasite/cell growth is reduced by half; SI, selectivity is calculated by the EC₅₀ value in HeLa cells divided by the EC₅₀ value obtained for the parasite). Note that In-2, In-5 and In-6 performed the best in these mice studies.

(B) Plots between IC₅₀ and EC₅₀ values obtained for malaria parasites showing high correlation between the two.

(C) Scatterplots showing the relationship between the SI obtained in the malaria parasite growth inhibition assays and the IC₅₀ values calculated from the PfPRs enzyme inhibition assays.

indexes of less than 20, probably due to their bioavailability compared with In-5 or In-2 (Table S6). The correlation between IC₅₀ and EC₅₀ for these QBI compounds showed high linkage, and these analyses therefore demonstrate utilities of PRSs as targets and the QBI series as leads (Figure 6B). Similarly, the relation between the SI (for mammalian cells) and the EC₅₀ values for *Plasmodium* parasite showed a clustering of the best performing QBIs In-1, In-2, In-4, In-5, In-6 and In-7 with SI

values of over ~500 over, and a poorer SI cluster with EC₅₀ values in the range of 1 and 350 nM (Figure 6C).

DISCUSSION

Developing strategies that act on key vulnerabilities within eukaryotic parasites is necessary for drugging human pathogens with novel therapeutics. To circumvent the arduous and

expensive routines of classical drug discovery, targeted drug discovery on common and conserved pathogen targets is gaining pace (Jain et al., 2014; Khare et al., 2016; Palencia et al., 2016; Sonoiki et al., 2016; Jain and Sharma, 2017; Jain et al., 2017). Natural compound derivatizations have provided many known drugs such as chloroquine and dihydro-artemisinin. Similarly, HF, a 6,7-dihalogenated derivative of the natural compound febrifugine is known to possess high anti-malarial potential; however, poor tolerability has prevented its clinical development (Hewitt et al., 1952). Numerous reports have suggested that aarSs represent attractive targets for the treatment of parasitic infections (Jain et al., 2014, 2015; Khan et al., 2014; Koh et al., 2012; Palencia et al., 2016; Pham et al., 2014; Sonoiki et al., 2016). Recently, Schreiber and co-workers identified BRD7929, a synthetic compound that targets the *P. falciparum* phenylalanyl-tRNA synthetase and offers single-dose clearance in mouse models (Kato et al., 2016). Similarly, Winzeler and co-workers had identified cladosporin as a nanomolar range drug that targets the malarial lysyl-tRNA synthetase (Hoepfner et al., 2012); a breakthrough that has now been extended to parasites such as *Loa loa* and *Schistosoma mansoni* (Sharma et al., 2016). In addition, Tavaborole, an FDA-approved anti-fungal that targets the editing domain of the leucyl-tRNA synthetase, is being marketed for the treatment of onychomycosis (Rock et al., 2007; Seiradake et al., 2009). Along with these successes, we here show that quinazolinone-based derivatives can serve as excellent candidate molecules for furthering drug development against a number of eukaryotic parasites that possess the essential and conserved housekeeping enzyme prolyl-tRNA synthetase. Our target-, cell-, and structure-based approaches validate PRSs from *Plasmodium*, *Toxoplasma*, *Leishmania*, *Cryptosporidium* and *Eimeria* as immensely druggable targets using quinazolinone-scaffold series. Validation of these parasite PRSs is supported through several lines of evidence, including: (1) *in vitro* target-based binding and PRS inhibition data, (2) parasite growth inhibition data for *Plasmodium*, *Toxoplasma*, *Leishmania* and *Cryptosporidium* parasites, (3) structural data that allow atomic resolution access to the binding determinants between PRSs and QBIs, and (4) *in vivo* mouse malaria infection model data that indicate clearance of infection using QBIs.

We note that identification of broadly active pan-protozoan scaffolds makes it feasible to individually tailor each derivative on a per parasite basis in order to optimize its drug-like properties. More significantly, the PRSs in each of these parasites provide a conserved, common target that can be utilized for screening new drug-like libraries with better ADME (absorption, distribution, metabolism, and excretion) properties. In addition, alternative analogs from this series with different pharmacological profiles might also be useful. Given that there are scarce resources for drug development against most neglected diseases, the validation of common druggable targets and shared, potent chemical scaffolds bodes (Jain et al., 2017) well for accelerating drug development against multiple human pathogens.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- QUANTIFICATION AND STATISTICAL ANALYSIS
- DATA AND SOFTWARE AVAILABILITY

ACCESSION NUMBERS

Coordinates and structure factors for ten structures have been deposited into PDB under accession codes PDB: 5XIF, 5XIG, 5XIH, 5XII, 5XIJ, 5XIK, 5XIL, 5XIO, 5XIP and 5XIQ.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, six tables, one movie and one data file and can be found with this article online at <http://dx.doi.org/10.1016/j.str.2017.07.015>.

AUTHOR CONTRIBUTIONS

V.J. performed protein purifications of the six PRSs, and all enzymatic and binding affinity assays. V.J. also performed the malarial parasite culture experiments and protein crystallization experiments. V.J. and M.Y. collected X-ray data and determined the crystal structures. P.G. helped V.J. in the protein purification of TgPRS, CpPRS, LmPRS and EtPRS. H.K., Y.O., N.H., and M.M. performed the chemical synthesis and the *in vivo* mice work. R.S.J. and C.D.H. performed the *Cryptosporidium* parasite growth inhibition experiments. B.T. and M.A.H. performed the *Toxoplasma* parasite growth inhibition experiments. F.T.C. supervised *Leishmania* parasite growth inhibition experiment. K.H. collected the CpPRS data at beamline I04 of Diamond Light Source. V.J. and A.S. designed the study and participated in data analyses. Primarily V.J. and A.S. wrote the manuscript but all authors discussed the results and edited the manuscript.

ACKNOWLEDGMENTS

ESRF BM14 beamline and DLS I04 beamline in collaboration with Division of Structural Biology (STRUBI, proposal MX14744), University of Oxford were used for X-ray data collection. Primarily, the research was supported by DBT OSRP grant PR6303 (to A.S.) and DBT grant PR13636 (to A.S. and M.Y.). A Senior Research Fellowship from DBT to V.J. is also acknowledged. A.S. is also supported by the JC Bose fellowship. Grants-in-Aid for Scientific Research (no. 16H03279) from MEXT (Japan) (to H.K.). The Platform Project for Supporting in Drug Discovery and Life Science Research from AMED (Japan) (to O.Y.). M.A.H. and B.T. were supported by the LabEx ParaFrap and the ERC Consolidator grant (no. 614880-Hosting TOXO). R.S.J. and C.D.H. were supported by a subcontract to grant OPP103258 from the Bill

and Melinda Gates Foundation. F.T.C. was supported by Swiss National Foundation, grant 310030.146187/1. The Wellcome Trust Center for Human Genetics is supported by the Wellcome Trust (grant 090532/Z/09/Z). We thank STRUBI, Oxford, and Prof. David Stuart for enabling access to DLS and STRUBI.

Received: April 9, 2017

Revised: June 2, 2017

Accepted: July 26, 2017

Published: August 31, 2017

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
Bacteria: E. coli BL21 (DE3)	EMD-millipore	69450-3
Chemicals, Peptides, and Recombinant Proteins		
Protein: PfPRS (254 – 746)	This paper	UniProt ID: Q8I5R7
Protein: TgPRS (334 – 830)	This paper	UniProt ID: S8G8I1
Protein: LmPRS (224 – 731)	This paper	UniProt ID: Q4QDS1
Protein: CpPRS (226 – 719)	This paper	UniProt ID: Q5CWN3
Protein: EtPRS (254 – 747)	This paper	UniProt ID: U6KWI1
Protein: HsPRS (1016 – 1512)	This paper	UniProt ID: P07814
Chemical: In-1	This paper	N/A
Chemical: In-2	This paper	N/A
Chemical: In-3	This paper	N/A
Chemical: In-4	This paper	N/A
Chemical: In-5	This paper	N/A
Chemical: In-6	This paper	N/A
Chemical: In-7	This paper	N/A
Chemical: In-8	This paper	N/A
Chemical: In-9	This paper	N/A
Chemical: Halofuginone	SIGMA	32481
Deposited Data		
TgPRS-apo	This paper	PDB: 5XIF
TgPRS-In-1-ANP	This paper	PDB: 5XIG
TgPRS-In-5-ANP	This paper	PDB: 5XIH
TgPRS-In-6-ANP	This paper	PDB: 5XII
TgPRS-In-9-ANP	This paper	PDB: 5XIJ
TgPRS-ThFF-ANP	This paper	PDB: 5XIK
TgPRS-HF-ANP	This paper	PDB: 5XIQ
CpPRS-HF-ANP	This paper	PDB: 5XIO
EtPRS-HF-ANP	This paper	PDB: 5XIP
LmPRS-apo	This paper	PDB: 5XIL
Experimental Models: Cell Lines		
Mouse: L929 fibroblast cells	ATCC	CCL-1
Human: HeLA cells	ATCC	CCL-2.2
Human: ileocecal adenocarcinoma cells	ATCC	CCL-244
Human: foreskin fibroblasts	ATCC	SCRC-1041
Experimental Models: Organisms/Strains		
Mouse: ICR	The Jackson Laboratory	009122
Recombinant DNA		
pETM41	EMBL	https://www.embl.de/pepcore/pepcore_services/cloning/choice_vector/ecoli/embl/popup_emblvectors/
pETM11	EMBL	https://www.embl.de/pepcore/pepcore_services/cloning/choice_vector/ecoli/embl/popup_emblvectors/
Software and Algorithms		
Prism	GraphPad	https://www.graphpad.com/scientific-software/prism/
Protein Thermal Shift™ Software	Thermofisher	https://www.thermofisher.com/order/catalog/product/4466037

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Illustrator CS6	Adobe	http://www.adobe.com/products/illustrator.html
PHENIX	Adams et al., 2010	https://www.phenix-online.org/
COOT	Emsley and Cowtan, 2004	https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/

CONTACT FOR REAGENT AND RESOURCE SHARING

Requests for further information, resources and reagents should be directed to the Lead Contact, Dr. Amit Sharma, Molecular Medicine group at the International Centre for Genetic Engineering and Biotechnology (ICGEB), Aruna Asaf Ali Marg, New Delhi, 110067, India. amitpsharma68@gmail.com

EXPERIMENTAL MODEL AND SUBJECT DETAILS**Bacterial Strains**

Recombinant proteins were expressed in *Escherichia coli* BL21 (DE3) cells grown in LB (Luria-Bertani) media at 37 °C.

Cell Lines

Mouse L929 fibroblasts were cultured in complete RPMI containing 10% fetal bovine serum (FBS) at 37 °C. HeLa cells were cultured in complete RPMI containing 10% FBS, 0.2% sodium bicarbonate and 50-mg/ml gentamycin at 37 °C. Human ileocecal adenocarcinoma (HCT-8) cells were cultured in complete RPMI containing 10% FBS at 37 °C. Human foreskin fibroblasts were cultured in DMEM supplemented with 10% FBS, 10 mM HEPES pH 7.2, 2 mM L-glutamine, and 50 µg/ml penicillin and streptomycin (Invitrogen) at 37 °C. Human bone marrow-derived macrophages (BMM) were cultured in complete RPMI containing 10% FBS at 37 °C.

METHOD DETAILS**Compounds Synthesis****Synthesis of compound B**

To a solution of compound A (Kikuchi et al., 2006) (282 mg, 0.931 mmol) in pyridine (2.5 mL) was added *p*-toluenesulfonyl chloride (532 mg, 2.79 mmol) at room temperature. After being stirred for 1.5 hours, the mixture was poured into 0.2 M hydrochloric acid and extracted with ethyl acetate three times. The organic layer was washed with saturated sodium bicarbonate solution and brine, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The residue was chromatographed over silica gel eluted by hexane-ethyl acetate (3:1) to give a tosylate (342 mg, yield 80%).

To a solution of 4-hydroxyquinazoline (30 mg, 0.205 mmol) in DMF (2.0 mL) was added potassium carbonate (113 mg, 0.818 mmol) at 0 °C. After 15 minutes, the tosylate of compound A (94 mg, 0.205 mmol) dissolved in DMF (1.0 mL) was added into the solution. After being stirred for 4 hours at 50 °C, the mixture was poured into water and extracted with ethyl acetate three times. The organic layer was washed with water and brine, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The residue was chromatographed over silica gel eluted by hexane-ethyl acetate (2:1) to give compound B (64 mg, yield 72%). Please see Figure S7.

Synthesis of Compound C

Compound B (60 mg, 0.139 mmol) was dissolved in 10% hydrogen chloride-methanol solution (2 mL). After being stirred for 2 hours at 50 °C, the mixture was concentrated *in vacuo*. The residue was dissolved in methanol (1 mL), and triethylamine (40 µL, 0.544 mmol) and di-*tert*-butyl carbonate (61 mg, 0.278 mmol) were added to the solution. After being stirred for 3 hours at room temperature, the mixture was poured into 0.2 M hydrochloric acid and extracted with ethyl acetate three times. The organic layer was washed with saturated sodium bicarbonate solution and brine, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The residue was chromatographed over silica gel eluted by hexane-ethyl acetate (1:2) to give compound C (47 mg, yield 87% (2 steps)). Please see Figure S7.

Synthesis of In-1

Compound C (30 mg, 0.078 mmol) was dissolved in dichloromethane (1 mL). Then, Dess-Martin periodinane (15wt% solution in dichloromethane) (0.33 mL, 0.116 mmol) and water (20 µL) were added to the solution at 0 °C. After being stirred for 1 hour, the mixture was poured into 2 M sodium hydroxide and extracted with ethyl acetate three times. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The residue was chromatographed over silica gel eluted by hexane-ethyl acetate (1:2) to give a ketone (24 mg, yield 80%).

The ketone (14 mg, 0.036 mmol) was dissolved in 10% hydrogen chloride-methanol solution (1 mL). After being stirred for 2 hours at room temperature, the mixture was concentrated *in vacuo*. The residue was chromatographed over silica gel eluted by chloroform-methanol (9:1) to give In-1 (10 mg, yield 92%) as dihydrochloride. Data for In-1: ¹H NMR (400 MHz, methanol-*d*₄) δ 9.34 (1H, br.s), 8.36 (1H, dd, *J* = 8.1, 1.4 Hz), 8.01 (1H, ddd, *J* = 8.3, 7.3, 1.4 Hz), 7.78 (1H, dd, *J* = 8.3, 1.0 Hz), 7.76 (1H, ddd, *J* = 8.1, 7.3,

1.0 Hz), 4.26 (2H, br.s), 3.26-3.30 (1H, m), 3.14-3.24 (1H, m), 2.99-3.10 (1H, m), 2.01-2.31 (4H, m), 1.79-1.91 (2H, m), 1.59-1.75 (2H, m); HRFABMS: m/z 286.1528 [M+H]⁺ (286.1556 calculated for C₁₆H₂₀O₂N₃).

Synthesis of In-2~7

In similar ways, In-2~7 were synthesized by using corresponding quinazoline derivatives instead of 4-hydroxyquinazoline.

Data for In-2: ¹H NMR (400 MHz, methanol-*d*₄) δ 9.60 (1H, br.s), 8.04 (1H, d, *J* = 6.7 Hz), 7.85-7.91 (2H, m), 4.26 (2H, br.s), 3.26-3.30 (1H, m), 3.14-3.24 (1H, m), 2.99-3.10 (1H, m), 2.01-2.31 (4H, m), 1.79-1.91 (2H, m), 1.59-1.75 (2H, m); HRFABMS: m/z 304.1478 [M+H]⁺ (304.1461 calculated for C₁₆H₁₉O₂N₃F).

Data for In-3: ¹H NMR (400 MHz, methanol-*d*₄) δ 9.71 (1H, br.s), 8.43 (1H, t, *J* = 7.2 Hz), 7.58-7.62 (2H, m), 4.27 (2H, br.s), 3.23-3.38 (1H, m), 3.17-3.20 (1H, m), 2.99-3.10 (1H, m), 2.07-2.29 (4H, m), 1.79-1.98 (2H, m), 1.54-1.70 (2H, m); HRFABMS: m/z 304.1469 [M+H]⁺ (304.1461 calculated for C₁₆H₁₉O₂N₃F).

Data for In-4: ¹H NMR (400 MHz, methanol-*d*₄) δ 9.71 (1H, br.s), 8.03 (1H, td, *J* = 8.4, 4.5 Hz), 7.62 (1H, d, *J* = 8.4 Hz), 7.50 (1H, dd, *J* = 10.1, 8.4 Hz), 4.24 (2H, br.s), 3.26-3.32 (1H, m), 3.17-3.23 (1H, m), 3.01-3.10 (1H, m), 2.06-2.28 (4H, m), 1.78-1.98 (2H, m), 1.64-1.74 (2H, m); HRFABMS: m/z 304.1473 [M+H]⁺ (304.1461 calculated for C₁₆H₁₉O₂N₃F).

Data for In-5: ¹H NMR (400 MHz, methanol-*d*₄) δ 9.50 (1H, br.s), 8.22 (1H, t, *J* = 8.8 Hz), 7.77 (1H, dd, *J* = 9.9, 6.6 Hz), 4.18-4.28 (2H, m), 3.23-3.34 (1H, m), 3.15-3.20 (1H, m), 3.00-3.09 (1H, m), 1.98-2.29 (4H, m), 1.80-1.90 (2H, m), 1.59-1.71 (2H, m); HRFABMS: m/z 322.1356 [M+H]⁺ (322.1367 calculated for C₁₆H₁₈O₂N₃F₂).

Data for In-6: ¹H NMR (600 MHz, methanol-*d*₄) δ 9.10 (1H, br.s), 8.40 (1H, d, *J* = 7.6 Hz), 7.63 (1H, d, *J* = 9.2 Hz), 4.14-4.24 (2H, m), 3.22-3.29 (1H, m), 3.14-3.20 (1H, m), 2.99-3.07 (1H, m), 2.23-2.29 (1H, m), 2.07-2.20 (2H, m), 1.96-2.05 (1H, m), 1.80-1.91 (2H, m), 1.59-1.71 (2H, m); HRFABMS: m/z 338.1068 [M+H]⁺ (338.1072 calculated for C₁₆H₁₈O₂N₃³⁵ClF).

Data for In-7: ¹H NMR (600 MHz, methanol-*d*₄) δ 9.07 (1H, br.s), 8.35 (1H, s), 8.09 (1H, s), 4.14-4.24 (2H, m), 3.22-3.30 (1H, m), 3.13-3.21 (1H, m), 2.97-3.06 (1H, m), 2.23-2.29 (1H, m), 2.07-2.20 (2H, m), 1.94-2.05 (1H, m), 1.80-1.91 (2H, m), 1.58-1.69 (2H, m); HRFABMS: m/z 398.0270 [M+H]⁺ (398.0271 calculated for C₁₆H₁₈O₂N₃⁷⁹Br³⁵Cl).

Synthesis of Compound D

To a solution of febrifugine (125 mg, 0.415 mmol) in methanol (3 mL) was added sodium borohydride (17 mg, 0.450 mmol) at 0 °C. After being stirred for 1 hour, the mixture was concentrated *in vacuo*. The residue was chromatographed over silica gel eluted by chloroform-methanol (4:1) to give compound D (86 mg, yield 68%).

Synthesis of Compounds E and F

To a solution of compound D (82 mg, 0.269 mmol) in methanol (3 mL) were added triethylamine (0.11 mL, 0.789 mmol) and benzyl chloroformate (0.11 mL, 0.771 mmol) at 0 °C. After being stirred for 8 hour, the mixture was poured into 0.2 M hydrochloric acid and extracted with ethyl acetate three times. The organic layer was washed with saturated sodium bicarbonate solution and brine, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The residue was chromatographed over silica gel eluted by ethyl acetate-methanol (9:1) to give the mixture of compounds E and F (92 mg). The mixture was subjected to recycle preparative HPLC (column, JAIGEL-GS310 (φ 20 mm x 500 mm, Japan Analytical Industry Co., Ltd.); solvent, chloroform) to give compound E (30 mg, 25%) and compound F (56 mg, 47%).

Synthesis of In-8 and 9

Compound E (28 mg, 0.064 mmol) and palladium hydroxide (5.0 mg) (20% on carbon) in 5% hydrogen chloride-methanol solution (2 mL) were stirred for 6 hours under hydrogen atmosphere. After filtration through a Celite pad, the filtrate was concentrated *in vacuo*. The residue was chromatographed over silica gel eluted by chloroform-methanol (3:1) to give compound E (20 mg, yield 82 %) as dihydrochloride. Data for In-8: ¹H NMR (400 MHz, methanol-*d*₄) δ 9.53 (1H, s), 8.39 (1H, dd, *J* = 8.3, 1.4 Hz), 8.07 (1H, td, *J* = 8.3, 1.4 Hz), 7.83 (1H, d, *J* = 8.3 Hz), 7.81 (1H, t, *J* = 8.3 Hz), 4.51 (1H, dd, *J* = 13.7, 3.0 Hz), 4.32-4.39 (1H, m), 4.02-4.10 (1H, dd, *J* = 13.7, 9.2 Hz), 3.67 (1H, dt, *J* = 9.9, 4.1 Hz), 3.26 (1H, dd, *J* = 8.8, 3.0 Hz), 2.99 (1H, dt, *J* = 12.4, 3.2 Hz), 1.94-2.02 (1H, m), 2.04-2.21 (3H, m), 1.79 (1H, tq, *J* = 12.1, 3.9 Hz), 1.58 (1H, dq, *J* = 12.4, 3.9 Hz); HRFABMS: m/z 304.1651 [M+H]⁺ (304.1661 calculated for C₁₆H₂₂O₃N₃).

In a similar way, In-9 was synthesized from compound F. Data for In-9: ¹H NMR (400 MHz, methanol-*d*₄) δ 9.43 (1H, s), 8.39 (1H, dd, *J* = 8.2, 1.0 Hz), 8.06 (1H, dt, *J* = 8.2, 1.4 Hz), 7.82 (1H, dd, *J* = 8.2, 1.0 Hz), 7.80 (1H, dt, *J* = 8.2, 1.4 Hz), 4.49 (1H, dd, *J* = 13.6, 2.8 Hz), 4.27-4.33 (1H, m), 3.61 (1H, dt, *J* = 10.1, 3.9 Hz), 2.98 (1H, dt, *J* = 12.8, 2.5 Hz), 2.03-2.13 (1H, m), 1.94-2.03 (1H, m), 1.67-1.80 (2H, m), 1.52-1.62 (1H, m); HRFABMS: m/z 304.1638 [M+H]⁺ (304.1661 calculated for C₁₆H₂₂O₃N₃).

Protein Production

All six PRSs (gene/protein details are provided in Table S2) were produced in *E. coli* BL-21 strain in accordance with the methods published (Jain et al., 2014). Briefly, Protein expression was induced by adding 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) to cells grown at 37 °C for 4 h, and for 20 h post-induction at 18 °C. Cells were harvested by centrifugation at 5,000 g for 15 min. Bacterial pellet was suspended in a buffer containing 50 mM Tris-HCl pH 8.0, 200 mM NaCl, 3 mM βME, 15% v/v glycerol, 0.1 mg ml⁻¹ lysozyme and EDTA free protease inhibitor cocktail (Roche). Cells were lysed by sonication and cleared by centrifugation at 20,000 g for 45 min. The cleared supernatant with MBP and His₆ tagged proteins were applied to amylose beads (NEB) or NiNTA beads (GE) and protein was eluted with buffer 50 mM Tris-HCl pH 8.0, 200 mM NaCl, 10 mM βME and 10 mM maltose or 250 mM Imidazole respectively. The eluted protein fractions were dialyzed against 30 mM HEPES pH 7.5, 20 mM NaCl, 1 mM DTT and 0.5 mM EDTA (buffer A). The protein was further purified by heparin chromatography (GE Healthcare) using NaCl gradients with buffer B containing 30 mM HEPES pH 7.5, 500 mM NaCl, 1 mM DTT and 0.5 mM EDTA. Protein peak was found at 40% buffer B. The

tags (MBP and His tag) was removed by incubating with TEV protease at 20 °C for 24 h. Cleaved PfPRS protein was concentrated using 10 kDa cutoff Centricon centrifugal device (Millipore) and purified by gel filtration chromatography on Superdex 200 column 16/60 GL (GE Healthcare) equilibrated with 20 mM HEPES pH 7.5, 200 mM NaCl and 2 mM DTT. Bovine serum albumin (66 kDa, Sigma) was used as a standard for the molecular mass estimation. Eluted fractions were checked by SDS-PAGE and pure ones pooled, concentrated and stored at -80 °C.

***P. falciparum* Cultures and Assays**

Plasmodium falciparum 3D7 was cultured in O⁺ erythrocytes in RPMI 1640 (Invitrogen, USA) supplemented with 4.5 mg ml⁻¹ glucose (Sigma, USA), 0.1 mM hypoxanthine (Invitrogen, USA), 25 mg ml⁻¹ gentamicin (Invitrogen, USA) and 0.5% AlbuMax-I (Invitrogen, USA), according to standard methods (Trager and Jensen, 2005). Parasites were sorbitol-treated in ring stages to maintain a synchronized culture as described (Tonkin et al., 2004). HF was dissolved in DMSO (Sigma) while Th-FF and nine QBIs were dissolved in double distilled water. *Plasmodium falciparum* was cultured in 96-well plates and synchronized at ring stages. At ~1% parasitemia and 4% hematocrit, inhibitor concentration ranges from 0.01 nM to 10 μM were incubated for 48 hours with the parasites. Growth was assayed by SYBR green-I (Molecular probes) DNA staining assays as described earlier (Smilkstein et al., 2004). Briefly, 100 μl SYBR green dye in 1 × concentration in lysis buffer supplemented with 0.1% saponin was added to each well. After 45 min incubation at 37 °C fluorescence was estimated using multi-well plate reader (Victor 3, Perkin Elmer) with excitation and emission wavelength bands centered at 485 and 530 nm respectively. Chloroquine was taken as a positive control and all experiments were in triplicates. The IC₅₀ values were obtained by plotting fluorescence values expressed in terms of percentage inhibition of parasite growth at each inhibitor concentration. Analyses were done using nonlinear regression analysis with GraphPad Prism 5 software. All data are shown for three replicates as means with standard errors.

***T. gondii* Parasite and Host Cell Cultures**

A type-I *T. gondii* reference laboratory strain RH-YFP was used for 50% effective concentration (EC₅₀) determination. The parasites were maintained by serial passage in human foreskin fibroblasts (HFF, ATCC® SCRC-1041) monolayer under tachyzoite growth conditions in DMEM (Invitrogen) supplemented with 10% heat inactivated FBS (Invitrogen), 10 mM HEPES pH 7.2, 2 mM L-glutamine, and 50 μg/ml penicillin and streptomycin (Invitrogen). *In vitro* inhibitory concentrations for Halofuginone and derivative compounds against the RH strain were determined by High Content Imaging assays. Briefly, HFFs were seeded at a density of 10,000 cells per well into 96-well plates and were allowed to grow and equilibrate for 24 hours at 37 °C to obtain sub-confluent monolayer. Cells were then infected with 4 × 10⁴ parasites/well. Invasion was synchronized by briefly centrifugating the plate at 400 rpm and placed at 37 °C for 2 hours. Then, infected cells were washed three times with PBS, followed immediately by addition of test compounds serially diluted from DMSO stocks in culture medium. Final DMSO concentrations ranged from 0.018 to 0.12%. Pyrimethamine was used as a positive control in all assays performed. The assay was run for 24 hours. Hoechst 33342 stain was then loaded on live cells/parasites at 5 μg/ml for 20 min. Three washes were applied before the cells and parasites were fixed with pre-warmed formaldehyde at 3.7% for 10 min at 37 °C. A mouse anti-GRA1/alexa 488 labeling (dilution 1/500) was used to monitor vacuole number. Images were acquired using the scanR system (Olympus, Germany). The 20X objective (N.A. 0.45) was used to collect images for the distinct fluorescence channels. Hoechst 33342 (ex. 360-370 nm, em. 420-460) and Alexa 488 (ex. 460-495, em. 510-550). Exposure time was 50 ms for the Hoechst channel, 100 ms for Alexa 488 the channel. Twenty fields per wells were imaged and analyzed using the scanR analysis module. Parasitophorous vacuoles were analyzed as following: a background subtraction was applied in all set images and then an intensity algorithm module was used with a minimum of 100 pixels size in order to segment the smallest vacuoles (one or two parasite). Parasite nuclei were discriminated from host nuclei by using an edge segmentation module. ScanR analysis module interface as in flow cytometry allow us to extract and display data as scatter plots and histograms. Using a 'gating' procedure we were able to hierarchically filter selected data points with precise boundaries (e.g. number of vacuoles vs. number of parasite/vacuoles). IC₅₀ values were determined by fitting dose response curve using the GraphPad Prism software and have been summarized in Figure 3D and Table S1.

***Leshmania* Major Survival Assays within Infected Macrophages**

A total of 5 × 10⁵ bone marrow-derived macrophages (BMM) were plated on coverslips (Thermo Scientific, Rochester, NY) in 24-well plates and allowed to adhere overnight (minimum 4H) in complete RPMI-10% FCS. The macrophages were infected with *L. major* (LV39 stain) at a 5 : 1 of parasites : macrophages ratio. Four hours later non-internalised extracellular parasites were removed by 3 rounds of washing. The infected macrophages were then incubated with 0.25 nM, 0.5 nM, 1 nM, 2 nM, 4 nM and 6 nM of HF for 16 hours. The coverslips were stained using Diff-Quik staining (Medion Diagnostics, Dudingen, Switzerland) according to the manufacturer's instructions. The frequency of infected macrophages and the number of intracellular parasites per 100 infected macrophages were determined by microscopy, counting 100 macrophages in triplicate coverslips, using blinded codes.

***C. parvum* Growth Inhibition Assays**

Intracellular *Cryptosporidium parvum* growth inhibition was assayed using a cell-based immunofluorescence technique as previously described (Besoff et al., 2013). *C. parvum* Iowa strain oocysts (Bunchgrass Farms, USA) were excysted by first treating with 10 mM HCl at 37 °C for 10 min, followed by a 2 mM solution of sodium taurocholate (Sigma) at 16 °C for 10 min. Oocysts were then diluted in RPMI-1640 medium (Invitrogen) supplemented with 10% heat inactivated FBS (Sigma), 120 U/mL penicillin and 120 μg/mL

streptomycin (ATCC) (culture media) and added at approximately 5500 oocysts per well to 100% confluent human ileocecal adenocarcinoma (HCT-8) cells (ATCC) grown in 384-well plates (BD Falcon). Compounds were added 3 h post-infection, and plates were incubated at 37 °C, 5% CO₂ for another 45 h. To prepare plates for immunofluorescence microscopy, three washes with 111 mM D-galactose were followed by fixation with 4% paraformaldehyde at room temperature for 15 min and permeabilization with 0.25% Triton X100 in PBS at 37 °C for 10 min. The cells were then washed three times with PBS containing 0.1% Tween 20, and blocked with 4% bovine serum albumin (BSA) (Sigma) in PBS at 37 °C for 2 h or 4 °C overnight. *Cryptosporidium parvum* vacuoles were stained at 37 °C for 1 h with 1.33 µg/mL of *Vicia villosa* lectin conjugated to fluorescein (Vector Laboratories), followed by staining nuclei with Hoechst 33258 (Anaspec) at 0.09 mM at 37 °C for another 15 min. The wells were then washed five times with PBS containing 0.1% Tween 20 before imaging using a Nikon Eclipse Ti2000 inverted epifluorescence microscope equipped with an EXi blue digital microscopy camera (QImaging, Canada) and NIS-Elements Advanced Research software version 4.30.01 (Nikon, USA). Using a 20X objective and a motorized stage, the software was programmed to take a three-by-three composite of images with 15% overlap after automatically focusing on approximately the center of each well. Image-J software version 1.48 (National Institutes of Health) was used to quantify nuclei and parasite numbers using previously developed macros and the batch function on exported.tif files (Bessoff et al., 2013). Dose response curves were then generated using GraphPad Prism software and have been depicted in Figure 3B.

Thermal Shift Assays

Thermal shift assays for PfPRS, TgPRS, CpPRS, EtPRS, LmPRS and HsPRS (Table S2) in presence of nine QBIs were performed as reported earlier (Jain et al., 2014; Jain et al., 2015). Briefly, purified proteins alone and/or with various drugs were heated from 25 to 99 °C at a rate of 1 °C min⁻¹ and fluorescence signals of the SYPRO® orange dye were monitored by StepOnePlus™ quantitative real-time PCR system (Life Technologies). Each curve is an average of three measurements and data were analyzed using Protein Thermal shift software (v1.3, Thermofisher) as shown in Figure 2B. Inhibitors and AMPPNP alone in assay buffers, along with no protein controls were used and flat lines were observed for these fluorescence readings across the temperatures.

Aminoacylation Assays

Complementary DNA oligonucleotides were purchased from Sigma with one primer containing the T7 RNA polymerase promoter followed by gene encoding the tRNA^{Pro} while the other primer encoded the complementary strand with promoter and the tRNA gene. These two oligos were mixed in equimolar ratios in an annealing buffer (10 mM Tris pH 8.0, 50 mM NaCl and 0.5 mM EDTA), heated to 95 °C and then slow cooled at room temperature. The annealed dsDNA was used as template for *in vitro* transcription reaction to produce tRNA^{Pro}, performed using the Ambion Maxiscript T7 *in-vitro* transcription kit. The enzymatic assays were done according to an earlier report (Cestari and Stuart, 2013). The standard aminoacylation reaction ingredients were aminoacylation buffer (30 mM HEPES buffer pH 7.4, 140 mM NaCl, 30 mM KCl, 40 mM MgCl₂), 1 mM DTT, 200 µM ATP, 2 U/ml inorganic pyrophosphatase (Sigma-Aldrich), 10 mM L-Pro (Sigma-Aldrich), 8 µM tRNA^{Pro} (each for *T. gondii*, *P. falciparum*, *C. parvum*, *E. tenella*, *L. major* and *H. sapiens*), and 0.4 µM of the six recombinant PRSs. The aminoacylation reactions were performed as before (Jain et al., 2015). For time course experiments for each of the six recombinant PRSs, 10 mM EDTA was added to reaction mixture at different time points (0, 2, 5, 8, 10, 20 and 30 min) and mixed on ice to stop the reaction. For PRSs inhibition, a reaction solution containing individual PRS (0.4 µM) was mixed with QBIs at 0.1 nM to 10 µM in 50 µl volume and incubated for ~20 min at 37 °C. Reactions were stopped as described above. All data are shown for three replicates as the means with the standard errors in Figure 2A and Table S3.

Cytotoxicity Assays

The cytotoxicity test of compounds was done by the colorimetric MTT assay technique (Mosmann, 1983). Mouse L929 cell suspension in RPMI- 1640 with 10% FBS (90 mL) was added in 96-well microplates at 1.8×10^4 cells/well. Then, a solution of test compounds dissolved in distilled water or 10% DMSO (10 mL) was added to each well. After incubation for 48 h at 37 °C under 5% CO₂, 2.5 mg/mL MTT solution (10 mL) was added to each well. The plate was incubated further 4 h. Then, the incubation medium was aspirated, and DMSO (100 mL) was added to solubilize the MTT formazan product. After mixing, absorbance at 570 and 655 nm were measured.

For human HeLa, cells were cultured in complete RPMI containing 10% fetal bovine serum, 0.2% sodium bicarbonate and 50 mg/ml gentamycin. Briefly, cells (10^4 cells/200 µl/well) were seeded into 96-well flat-bottom tissue-culture plates in complete culture medium. Drug solutions were added after overnight seeding and incubated for 24 h in a humidified atmosphere at 37 °C and in 5% CO₂. DMSO (final concentration 10%) was added as positive control. An aliquot of stock solution of MTT (5 mg/ml in 1x phosphate-buffered saline) was added at 20 µl per well, and incubated for another 4 h. After spinning the plate at 1500 rpm for 5 min, supernatants were removed and 100 µl of the stop agent DMSO added. Formation of formazan (chromogenic product from MTT reduction) - an index of survival, was measured at 570 nm. The EC₅₀ values were estimated from a dose response curve.

Crystallization

Highly pure proteins (PfPRS, TgPRS, CpPRS, EtPRS, LmPRS and HsPRS) were stored in 15 mM HEPES pH 7.5, 200 mM NaCl, 3 mM DTT at 193 K. Before crystallization, 3 mM inhibitors and 5 mM AMPPNP (also called as ANP in this paper) were added to protein solutions and incubated at 4 °C for 10 min. Crystals were obtained using vapor diffusion method in hanging drops. The TTP Labtech

Mosquito® was employed to mix 100 nanolitres of PRSs alone or in complex (AMPPNP and HF or QBIs) with 100 nl of well solutions from commercially available crystal screens- Hampton Research and Molecular Dimensions. Diffraction quality crystals for TgPRS-apo, TgPRS-HF-AMPPNP, TgPRS-In-1-AMPPNP, TgPRS-In-5-AMPPNP, TgPRS-In-6-AMPPNP, TgPRS-In-9-AMPPNP, TgPRS-ThFF-AMPPNP, EtPRS-apo, EtPRS-HF-AMPPNP, CpPRS-HF-AMPPNP, LmPRS-apo were obtained with the well solution containing different constituents summarized in [Table S4](#).

Data Collection and Structure Determination

X-ray diffraction data were either collected at 100 K at ESRF BM-14 beamline, Grenoble or at Diamond Light Source (DLS), UK. Summary of the data collections is available in [Table S5](#). The diffraction images were processed and scaled with HKL-2000 program suite or using software at DLS. Structures were solved by molecular replacement in PHASER ([McCoy et al., 2007](#)) using PfPRS-apo structure ([Jain et al., 2014](#)) (PDB code: 4TWA) as the template. All models were initially refined using REFMAC5 ([Murshudov et al., 2011](#)) and completed with *phenix.refine* in PHENIX ([Adams et al., 2010](#)). X-ray refinement restraint parameters were generated for QBI inhibitors using Sketcher program in CCP4 Suite ([Winn et al., 2011](#)). QBIs, AMPPNP, Mg^{2+} , Zn^{2+} and water molecules were added into the electron density maps using COOT ([Emsley and Cowtan, 2004](#)). The quality of the final models and bound ligands was verified using composite simulated annealing omit maps. The occupancies of bound ligands and alternate conformations of protein residues were refined and confirmed using omit maps. Final model quality was analyzed using MolProbity ([Chen et al., 2010](#)). Statistics for data collection and structure refinements are given in [Table S5](#). All structural superposition and preparation of figures was done using UCSF Chimera ([Pettersen et al., 2004](#)) and PyMOL (<http://www.pymol.org>). The atomic coordinates and structure factors for the ten structures have been deposited into Protein Data Bank with accession codes- 5XIF, 5XIG, 5XIH, 5XII, 5XIJ, 5XIK, 5XIL, 5XIO, 5XIP, 5XIQ.

In Vivo Anti-Malarial Assays in Mice

QBI efficacy was determined against rodent malaria-derived *P. berghei* NK65 according to the 4-days suppressive test ([Peters, 1975](#)). Male ICR mice of weight 18~20 g were inoculated with 10^6 parasitized red blood cells intravenously. Test compounds were suspended in 5% gum Arabic (emulsifier), and orally administered to the mice two hours after infection (Day 0). Test compounds were successively administered to the mice once a day for 3 consecutive days (days 1~3). The day after the last treatment (day 4), blood was obtained from the tail vein of the infected mice. Parasitemia was determined using thin blood films made from the blood. The ED_{50} , LD_{50} and TI values were estimated from dose response curves and are summarized in [Figure 6A](#) and [Table S6](#).

Ethical Statement

This study was performed in accordance with the guidelines for the 'Animal Care and Use of' at Otsuka Pharmaceutical Co., Ltd. which were reviewed and approved by Japan Health Sciences Foundation ethics committee, Japan with identification number: 15-033. ICGEB has Institutional approvals for usage of anonymized human red blood cells (erythrocytes) obtained from the Rotary blood bank, New Delhi, India. All other cell lines used in this study are commercially available.

QUANTIFICATION AND STATISTICAL ANALYSIS

Results of replicate experiments were tabulated in Prism, which was used to calculate standard deviations, IC_{50} and EC_{50} . Measurements were made in triplicate at least ($n=3$), as indicated in figure legends and the method details.

DATA AND SOFTWARE AVAILABILITY

Protein structures and data upon which they rest have been deposited to the Protein Data Bank. The accession numbers for TgPRS-apo, TgPRS-In-1-ANP, TgPRS-In-5-ANP, TgPRS-In-6-ANP, TgPRS-In-9-ANP, TgPRS-ThFF-ANP, TgPRS-HF-ANP, CpPRS-HF-ANP and EtPRS-HF-ANP are reported in this paper as PDB: 5XIF, 5XIG, 5XIH, 5XII, 5XIJ, 5XIK, 5XIQ, 5XIO, 5XIP and 5XIL respectively.