ACS Infectious Diseases

pubs.acs.org/journal/aidcbc

Article

# Repositioning and Characterization of 1-(Pyridin-4-yl)pyrrolidin-2-one Derivatives as *Plasmodium* Cytoplasmic Prolyl-tRNA Synthetase Inhibitors

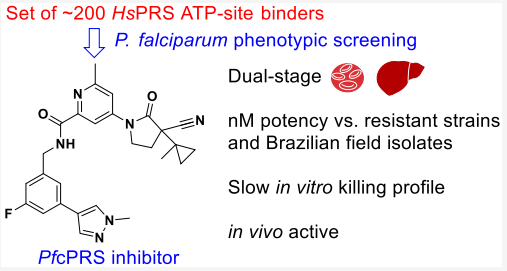
Masanori Okaniwa, Akira Shibata, Atsuko Ochida, Yuichiro Akao, Karen L. White, David M. Shackleford, Sandra Duffy, Leonardo Lucantoni, Sumanta Dey, Josefine Striepen, Tomas Yeo, Sachel Mok, Anna Caroline C. Aguiar, Angelika Sturm, Benigno Crespo, Laura M. Sanz, Alisje Churchyard, Jake Baum, Dhelio B. Pereira, Rafael V. C. Guido, Koen J. Dechering, Sergio Wittlin, Anne-Catrin Uhlemann, David A. Fidock, Jacquin C. Niles, Vicky M. Avery, Susan A. Charman, and Benoît Laleu*

Cite This: *ACS Infect. Dis.* 2021, 7, 1680–1689

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

**ABSTRACT:** Prolyl-tRNA synthetase (PRS) is a clinically validated antimalarial target. Screening of a set of PRS ATP-site binders, initially designed for human indications, led to identification of 1-(pyridin-4-yl)pyrrolidin-2-one derivatives representing a novel antimalarial scaffold. Evidence designates cytoplasmic PRS as the drug target. The frontrunner **1** and its active enantiomer **1-*S*** exhibited low-double-digit nanomolar activity against resistant *Plasmodium falciparum* (*Pf*) laboratory strains and development of liver schizonts. No cross-resistance with strains resistant to other known antimalarials was noted. In addition, a similar level of growth inhibition was observed against clinical field isolates of *Pf* and *P. vivax*. The slow killing profile and the relative high propensity to develop resistance *in vitro* (minimum inoculum resistance of $8 \times 10^5$ parasites at a selection pressure of $3 \times IC_{50}$) constitute unfavorable features for treatment of malaria. However, potent blood stage and antischizontal activity are compelling for causal prophylaxis which does not require fast onset of action. Achieving sufficient on-target selectivity appears to be particularly challenging and should be the primary focus during the next steps of optimization of this chemical series. Encouraging preliminary off-target profile and oral efficacy in a humanized murine model of *Pf* malaria allowed us to conclude that 1-(pyridin-4-yl)pyrrolidin-2-one derivatives represent a promising starting point for the identification of novel antimalarial prophylactic agents that selectively target *Plasmodium* PRS.




**KEYWORDS:** *malaria, Plasmodium, prolyl-tRNA synthetase, PRS*



Malaria remains a global scourge with 229 million cases of malaria and 409 000 deaths reported in 2019.[1] Human malaria is caused by five different species of parasites belonging to the *Plasmodium* genus. *Plasmodium falciparum* (*Pf*) and *Plasmodium vivax* (*Pv*) are the most prevalent species, the former being the most lethal. Increasing concerns of resistance to current drugs, including artemisinin combination therapies,[2,3] underscore the urgent need for novel antimalarials.[4,5] In that regard, focused target candidate and product profiles have been recently defined.[6]

The natural product febrifugine, with proven antimalarial activity in human,[7,8] has been shown to kill parasites through cytoplasmic prolyl-tRNA synthetase (cPRS) inhibition, demonstrating its clinical validation as an antimalarial target.[9] Febrifugine and its synthetic analogues, such as halofuginone,[10] (Figure 1) had unacceptable side effects precluding their further development but not detracting from the clinical relevance of this antimalarial target.[8,11] In addition, this chemical class was found to act through a proline-competitive binding mode[9] associated with a deleterious phenotypic drug resistance mechanism via accumulation of *L*-proline;[12] hence, it is necessary to explore alternative approaches to inhibit this enzyme. Allosteric inhibitors of *Pf*PRS were reported to provide high selectivity against the human orthologue (*Hs*PRS), but *in vitro* potency remained modest.[13] Recently, Takeda reported three novel chemotypes as *Hs*PRS inhibitors that were shown to act through competitive binding in the ATP site.[14−16] High structural homology between *Hs*PRS and

**Received:** January 13, 2021
**Published:** April 30, 2021

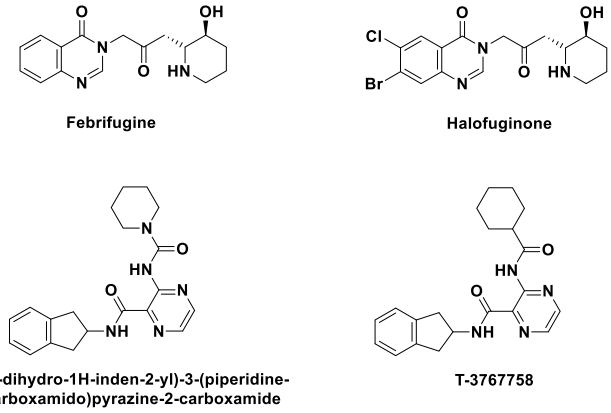



**Figure 1.** Chemical structures of febrifugine, halofuginone, 2,3-dihydro-1*H*-inden-2-yl)-3-(piperidine-1-carboxamido)pyrazine-2-carboxamide and T-3767758.

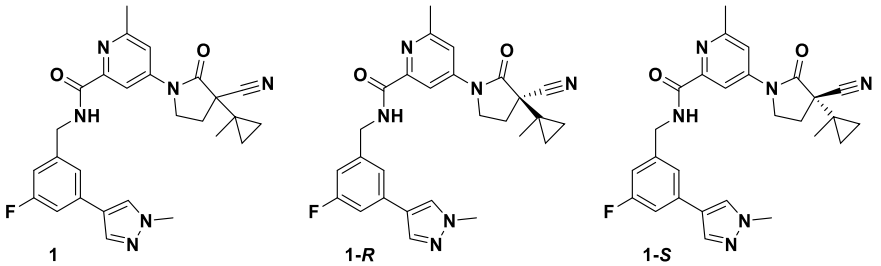
*Pf*PRS[17,18] offers an avenue to reposition *Hs*PRS inhibitors for malaria but poses challenges with respect to selectivity over the human orthologue. In that regard, *N*-(2,3-dihydro-1*H*-inden-2-yl)-3-(piperidine-1-carboxamido)pyrazine-2-carboxamide, the most potent antiplasmodium molecule (Dd2 $IC_{50}$ 63 nM) from a recent patent application,[19] is a very close urea analogue of T-3767758, previously reported as *Hs*PRS ATP-site binder[14] (Figure 1). This compound was shown to exert its activity in a proline-uncompetitive fashion with no indication regarding selectivity over *Hs*PRS.

Herein, we report the identification of 1-(pyridin-4-yl)pyrrolidin-2-one derivatives, a novel antimalarial class, through phenotypic screening of a set of *Hs*PRS ATP-site binders. cPRS appears as the likely antimalarial target of this chemotype. The frontrunner **1** and its active enantiomer **1-*S*** exhibited low double-digit nanomolar activity against all laboratory strains tested, *Pf*/*Pv* clinical field isolates, and developing *Pf* liver schizonts. In addition, *in vitro Pf* killing rate and resistance profile associated with this chemotype were obtained. Finally, both compounds demonstrated *in vivo* oral efficacy in a humanized SCID malaria model.

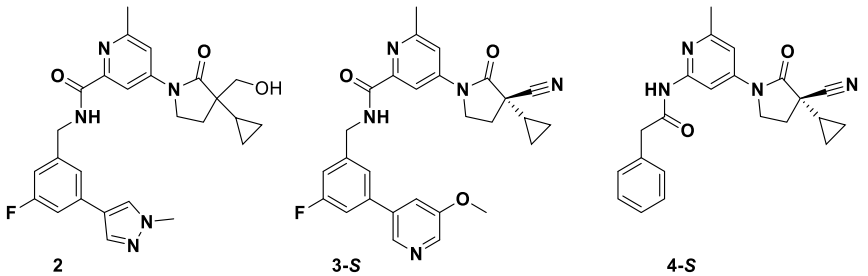
## RESULTS

**Identification of Novel Potent Antimalarials with Dual-Stage Activity.** Approximately 200 *Hs*PRS ATP-site binders from a Takeda proprietary library[20,21] were screened in a phenotypic *Pf* growth inhibition assay[22] and against HEK293 (human embryonic kidney) cell line to assess intraerythrocytic antiplasmodium activity and mammalian cytotoxicity, respectively. Results highlighted 1-(pyridin-4-yl)pyrrolidin-2-one derivatives as demonstrating attractive potency and acceptable selectivity index (SI > 50) against HEK293 cells.

Four representative compounds were nominated for resynthesis (Figure 2). As one of the most potent molecules presenting a SI > 50, compound **1** was selected as the frontrunner. Its attractive profile was confirmed from fresh solid material (*Pf*3D7 $IC_{50}$ = 10 nM, HEK293 $CC_{50}$ = 769 nM). The screening set provided by Takeda was composed of




| | 1 | 1-*R* | 1-*S* |
|---|---|---|---|
| *Pf*3D7 $IC_{50}$ (μM)[a] | 0.010[d] | 4.000 | 0.009 |
| HEK293 $CC_{50}$ (μM)[b] | 0.769[d] | >40 | 0.956 |
| Protein synthesis[c] $IC_{50}$ (μM) | 0.360 | ND | ND |




| | 2 | 3-*S* | 4-*S* |
|---|---|---|---|
| *Pf*3D7 $IC_{50}$ (μM)[a] | 0.879 | 0.149[d] | 0.058[e] |
| HEK293 $CC_{50}$ (μM)[b] | >20 | 2.357[d] | 0.957[e] |
| Protein synthesis[c] $IC_{50}$ (μM) | 7.700 | 0.670 | 0.880 |

**Figure 2.** Representative compounds of the 1-(pyridin-4-yl)pyrrolidin-2-one scaffold. [a]72 h DAPI growth inhibition assay, $N = 1$, duplicate points (same biological replicate). Pyrimethamine, chloroquine, artesunate, dihydroartemisinin, and puromycin were used as internal controls (mean $IC_{50}$ = 0.005; 0.023; 0.0005; 0.0002; 0.091 μM, respectively). [b]72 h assay, $N = 1$, duplicate points. [c]T-REx 293 cells transfected with plasmid encoding proline-rich peptide, $N = 2$, duplicate points; ND = not determined. [d]$N = 2$, duplicate points. [e]Mean values, $N = 4$, duplicate points.


https://doi.org/10.1021/acsinfecdis.1c00020
ACS Infect. Dis. 2021, 7, 1680−1689

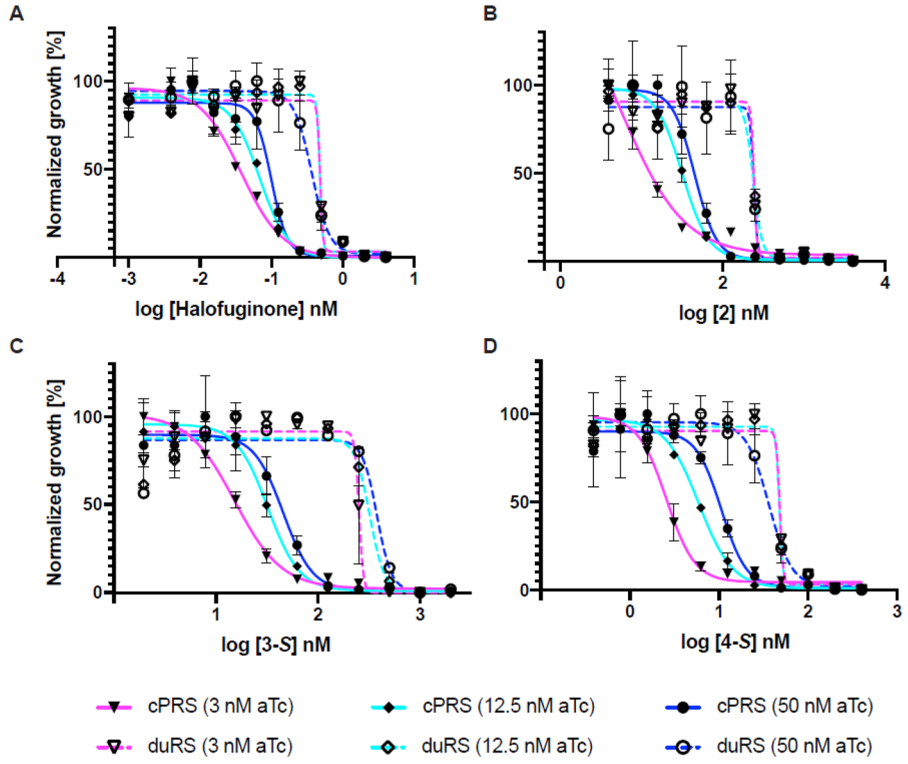



**Figure 3.** Dose−response curves for compounds incubated for 72 h with cPRS cKD parasites at low or higher aTc concentrations. [a]Values are mean ± SEM (error bars). (A) Halofuginone as positive control, (B) compound **2**, (C) compound **3-*S***, (D) compound **4-*S***.

racemates and enantiopure compounds, the latter presenting exclusively the *S* stereochemistry. Although this information was not available initially, enantiopreference was suspected. To confirm this suspicion, chiral separation was performed on compound **1**. The respective potencies of the two enantiomeric forms **1-*R*** (+) and **1-*S*** (−) revealed that the antiplasmodium activity was associated almost entirely with the *S* enantiomer (Figure 2). To maximize the chemical diversity within the selected set of molecules, **2** was chosen as a close structural analogue of **1**. Polarity was introduced by replacing the alkylnitrile with a primary alcohol to alter the physicochemical properties. Compound **3-*S*** highlights the scope for modifications at the heterocyclic moiety attached to the benzylic group. Compound **4-*S*** harbors an anilide instead of the carboxamide present in the other molecules and has no substituents on the benzylic group. These modifications were well tolerated without any deleterious impact on selectivity against mammalian cells. This small set of compounds can only provide very preliminary observations. Bioassays guided structural−activity relationships on a larger number of compounds initially designed as *Hs*PRS inhibitors, and interactive design of new molecules is required to expand upon these provisional observations.

The most potent compound **1-*S*** was selected for *Pf* life-cycle profiling. **1-*S*** potently inhibited the formation of *Pf* liver schizonts[23] ($IC_{50}$s of 13 and 9 nM against NF54 and NF175 strains, respectively, Figure S1) In contrast, no activity was evident against stage V gametocytes nor gamete formation based on a 1 $\mu$M incubation in the *Pf* male/female gamete formation assay.[24]

**Evidence Supports Cytoplasmic PRS as the Antimalarial Target.** *Pf* expresses two PRS enzymes: a cytoplasmic prolyl-tRNA synthetase (cPRS) and an apicoplast prolyl-tRNA synthetase (aPRS).[25] Compounds **2**, **3-*S***, and **4-*S*** were tested against *Pf* lines with an anhydrotetracycline (aTc)-inducible gene expression system to conditionally knockdown (cKD)[26] expression of cPRS or aPRS, and an unrelated dispensable reference target, tRNA dihydrouridine synthase (duRS; PF3D7_1408900). Using this inducible system, the regulated target protein level is highest at 50 nM aTc and reduced upon lowering aTc concentration. This allows profiling changes in parasite sensitivity to compounds after knockdown of putative target protein expression. When a target protein's function is inhibited by a test compound, reducing its expression level is expected to result in hypersensitivity to the compound. No such hypersensitization is expected, however, if a nontarget protein's expression is similarly titrated. Halofuginone, a known cPRS inhibitor,[9] was used as a positive control to benchmark relative responses of the cPRS, aPRS, and duRS cKD lines (Figure S2 and Table S1). Halofuginone inhibited the aPRS and duRS cKD lines with similar $IC_{50}$ values at all aTc concentrations. In contrast, at 50 nM aTc (maximum possible cPRS expression), the cPRS cKD line was more sensitive to halofuginone than the aPRS and duRS cKD lines. This suggests that modification of the cPRS locus to achieve regulated expression most likely resulted in reduced maximal cPRS expression[26] in the cPRS cKD line relative to the aPRS and duRS control lines, which express cPRS from its native locus. As cPRS expression levels were reduced further by lowering aTc concentration, the cPRS cKD line became even more sensitive to halofuginone (Figure 3 and Table S1). The basal and increasing hypersensitivity to halofuginone seen as aTc concentration is lowered for the cPRS cKD line, but not for the aPRS and duRS cKD lines, are consistent with the known inhibition of cPRS by halofuginone. Specifically, as cPRS levels decrease, lower halofuginone


https://doi.org/10.1021/acsinfecdis.1c00020
ACS Infect. Dis. 2021, 7, 1680−1689

concentrations are required to inhibit cPRS activity to cause parasite death. Having validated the cPRS cKD line, compounds **2**, **3-*S***, and **4-*S*** were evaluated. As with halofuginone, only the cPRS cKD line displayed basal and aTc-dependent hypersensitivity (Figures 3 and S2, $IC_{50}$ values reported in Table S1). Overall, these data support cytoplasmic PRS as the antimalarial target for this series of compounds.

**Human Cellular Protein Synthesis Inhibition Assay.** After obtaining further confidence that this chemotype exerts its antiplasmodium activity through inhibition of PRS, we focused our attention on the selectivity against the human orthologue. A protein synthesis inhibition assay previously established to monitor the intracellular synthesis of artificial proline-rich protein[14] was used to assess the effect due to inhibition of *Hs*PRS for compounds **1**, **2**, **3-*S***, **4-*S***. Data are included in Figure 2. As these compounds were initially designed as *Hs*PRS inhibitors, the observed selectivity at the cellular level in favor of antimalarial activity is encouraging for **1** and **4-*S*** (36- and 15-fold, respectively). On the basis of the data gathered, **1** was selected for antimalarial profiling to further evaluate the potential of repositioning this chemical series for malaria.

**Activity against Resistant Laboratory Strains of Malaria and Clinical Field Isolates.** Minimizing the risk of resistance is critical for development of novel malaria therapeutic agents. In that regard, pre-existing mutations can cause lack of correlation with potencies observed *in vitro* against sensitive parasite strains. Consequently, **1** was tested against various laboratory-resistant *Pf* strains to ensure activity against parasites resistant to historical antimalarial drugs.[27] Encouragingly, full activity was maintained against all resistant strains tested, irrespective of their resistance profile and geographical origin, with respect to the sensitive strain NF54 (Table 1). At the same time, no cross-resistance was observed with representative antimalarial agents currently in development[4] (Table S2).

In addition, the active enantiomer **1-*S*** was tested against *Pf* and *Pv* Brazilian clinical field isolates[28] and demonstrated potent parasiticidal activity with median $IC_{50}$ values of 9 and 15 nM, respectively (Figure S3, Table S3). Although the number of isolates tested was modest, these data suggest equipotency against the two most prevalent species of human malaria.

**Table 1. *In Vitro* Intraerythrocytic Antimalarial Activity of 1 against Laboratory Strains (Chloroquine and Artesunate as Controls)**

| laboratory strains | mutated loci | compound **1** $IC_{50}$ ($\mu$M)[a] | chloroquine $IC_{50}$ ($\mu$M)[a] | artesunate $IC_{50}$ ($\mu$M)[a] |
|---|---|---|---|---|
| NF54[b] | - | **0.018** | 0.008 | 0.004 |
| Dd2 | *Pf*crt, *Pf*mdr1, *Pf*dhfr, *Pf*dhps | **0.024** | 0.157 | 0.006 |
| K1 | *Pf*crt, *Pf*mdr1, *Pf*dhfr, *Pf*dhps | **0.020** | 0.202 | 0.002 |
| 7G8 | *Pf*crt, *Pf*mdr1, *Pf*dhfr, *Pf*dhps | **0.018** | 0.079 | 0.002 |
| TM90C2b | *Pf*crt, *Pf*mdr1, *Pf*dhfr, *Pf*dhps, $Pfcytb_{Q0}$ | **0.017** | 0.135 | 0.005 |
| Cam3.I | *Pf*crt, *Pf*mdr1, *Pf*dhfr, *Pf*dhps, *Pf*kelch13 | **0.015** | 0.180 | 0.011 |

[a]72 h [$^3$H]hypoxanthine incorporation assay, mean values from two independent biological replicates (variations between individual values did not exceed 11%). [b]Sensitive strain.

***In Vitro* Killing Profile.** To further characterize the antimalarial profile associated with this chemotype, the *in vitro* killing profile of **1** was determined in a parasite reduction ratio (PRR) assay. This assay uses a limiting dilution technique to quantify the number of *Pf* parasites that remain viable after drug treatment.[29] Data are depicted in Figure 4 with comparison to antimalarial reference drugs covering a broad range of different speed of kill profiles. Compound **1** was shown to be parasiticidal with a very slow killing profile (slower than atovaquone).

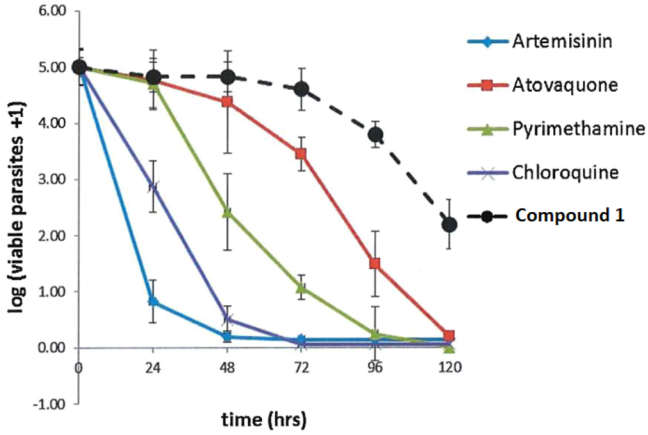



**Figure 4.** *In vitro* killing profile of **1** compared with reference antimalarial drugs (all tested at 10× their respective $IC_{50}$s).

***In Vitro* Resistance Profile.** To assess the *in vitro* resistance profile of **1**, minimum inoculum resistance (MIR) selections were set up with a selection pressure of 3 × $IC_{50}$. The first selection had a starting inoculum of 2 × $10^5$ *Pf* parasites in 24 replicates to give a parasite inoculum range from 2 × $10^5$ (single replicate) to 4.8 × $10^6$ (cumulation of all replicates). A second separate selection in parallel was set up with a starting inoculum of 2 × $10^7$ *Pf* parasites in triplicate for a higher inoculum range of 2 × $10^7$ to 6 × $10^7$ parasites. Parasites recrudesced on day 21 in the first lower starting inoculum selection in 6 of the 24 wells and on day 18 in all three wells of the second higher inoculum selection. Therefore, the MIR of **1** was determined to be 8 × $10^5$ (4.8 × $10^6$ divided by the 6 positive replicates). In contrast, the positive control DSM265,[30] which inhibits DHODH, never recrudesced within the smaller selection size (2 × $10^5$ parasites) and recrudesced within the larger size (2 × $10^7$ parasites) on day 25 in all three wells, giving it a MIR value of 2 × $10^7$.

To confirm resistance, resistant bulk cultures from both selections were assayed, revealing a small (3- to 4-fold) increase in $IC_{50}$ values (Figure S4). Whole genome sequencing performed on four **1**-resistant clones showed copy number variations (CNV) with a 2-fold amplification of a 63 kb region on chromosome 12 (Tables S4−S5). The position and range of the amplification between the selection at $10^5$ parasites (A2, C4, D1) and the one at $10^7$ (D4) varied slightly, yet in all four cases, the amplified region included cPRS (PF3D7_1213800) (Figure S5). This discovery reinforced confidence in cPRS as a resistance determinant


https://doi.org/10.1021/acsinfecdis.1c00020
ACS Infect. Dis. 2021, 7, 1680−1689

and likely target of this compound. No single nucleotide polymorphisms (SNPs) were found in these selections.

***In Vitro* DMPK and Physicochemical Properties.** The *in vitro* DMPK (drug metabolism and pharmacokinetics) and physicochemical properties of this chemical series were then explored. Representative 1-(pyridin-4-yl)pyrrolidin-2-one derivatives were tested for oxidative metabolism in human and rat liver microsomes, and aqueous solubility was measured (Table 2). Compounds showed low to moderate microsomal stability in both species studied, explainable by several metabolic hot spots (*vide infra* metabolite identification experiments) and a concomitant contribution of a nonoptimal LogD. The latter is corroborated by the higher microsomal stability exhibited by **2**. Poor aqueous solubility at pH 6.5 was obtained for all compounds and highlights another parameter to be improved for this chemotype.

**Table 2. *In Vitro* DMPK and Physicochemical Properties for Selected Compounds**

| compound | CL h-LM[a] (μL/min/mg protein) | CL r-LM[b] (μL/min/mg protein) | solubility[c] (μg/mL) | LogD[d] |
|---|---|---|---|---|
| **1** | 55 | 39 | <1.6 | 4.1 |
| **2** | 41 | 11 | 3.1−6.3 | 3.2 |
| **3-S** | 249 | 79 | <1.6 | 4.2 |
| **4-S** | 106 | 25 | 6.3−12.5 | 3.5 |

[a]Intrinsic clearance in human liver microsomes. [b]Intrinsic clearance in rat liver microsomes. [c]Aqueous kinetic solubility measured at pH = 6.5. [d]Distribution coefficient estimation at pH 7.4 using chromatography

On the basis of these data and antiplasmodium potency, **1** was chosen for further *in vitro* DMPK profiling (Table 3). Given the very poor solubility of **1** under near neutral conditions, we cannot rule out the possibility of solubility-related issues in the different *in vitro* assays; however, the data provide a general indication of further liabilities that need to be addressed for the series. Metabolic stability data were collected from mouse liver microsomes prior to conducting an *in vivo* efficacy proof-of-principle in a murine malaria model. **1** was revealed to be more stable in mouse hepatocytes than in mouse microsomes. The basis for this result is not currently understood and would require further investigation. Studies using Caco-2 cell monolayers demonstrated relatively good permeability, with no evidence for efflux transport (apical to basolateral $P_{app}$ of 22 × $10^6$ cm/s). In spite of the potential for solubility-related issues in the CYP inhibition assay with **1**, the inhibition of CYP2C9 and CYP3A4 indicates that further optimization might be necessary.

**Metabolite Identification and Glutathione Trapping Experiments.** To assess potential reactive metabolite formation and evaluate potential liabilities with respect to the alkylnitrile moiety, a comprehensive metabolite and glutathione (GSH) adduct search was conducted on **1** following incubation with human liver microsomes (Figure S6). *N*-Demethylation of the pyrazole was the predominant metabolite (based only on relative peak area as authentic metabolite standards were not available and therefore did not allow estimation of the percent contribution of the metabolites) followed by aliphatic hydroxylation at the pyrrolidinone moiety and *N*-dealkylation cleavage of the pyrazole-benzyl moiety. Remarkably, the nitrile moiety appeared to be particularly metabolically stable with no hydrolysis observed. Traces of a putative GSH conjugation product were observed; however, its MS/MS spectrum was too weak for structure confirmation.

**Rat *In Vivo* Pharmacokinetics.** To get a better understanding of the PK properties, the *in vivo* pharmacokinetic profile of **1** was assessed in rats (Figure 5, Table 4).

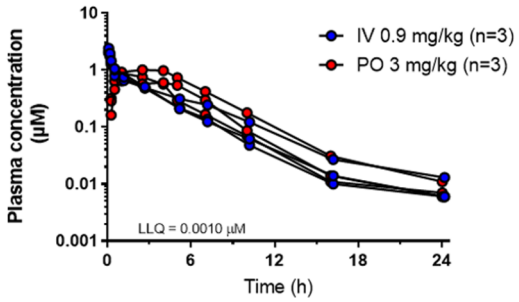



**Figure 5.** Plasma concentration versus time profiles for **1** in rats ($n$ = 3 animals per dose group). [a]IV vehicle: 5% DMSO in 1% Solutol HS15 (in saline), 1 mL per rat; PO vehicle: 0.5% HPMC/0.4% Tween 80 aqueous suspension, 5 mL/kg.

Following intravenous (IV) administration, plasma concentrations declined with a terminal half-life ($t_{1/2}$) of ∼9 h. A relatively low blood clearance of 12 mL/min/kg and a volume of distribution of 3.2 L/kg were estimated from the plasma data (rat whole blood-to-plasma ratio = 0.61). Given that binding to plasma proteins and to the *in vitro* test matrices (microsomes and hepatocytes) were not assessed, no attempts were made to relate the *in vitro* and *in vivo* intrinsic clearance values. Unchanged compound was not detected in urine, suggesting that direct urinary excretion did not contribute significantly to elimination and hepatic metabolism was the most likely basis for the observed *in vivo* clearance.

Following oral administration (PO), the half-life was comparable to that observed after IV dosing. Oral bioavailability (F) ranged from 30 to 50%. Noting that the *in vivo* blood clearance was equivalent to <20% of the nominal rat hepatic blood flow, it seems unlikely that hepatic first-pass elimination is the only factor limiting oral bioavailability. It is also unlikely that absorption is limited by permeability since the compound exhibited good membrane permeability across Caco-2 cell monolayers. Because of poor aqueous solubility, incomplete dissolution within the gastrointestinal tract may be a contributing factor to the somewhat limited bioavailability.

**Table 3. Additional *In Vitro* DMPK Characterization of 1**

| CL m-LM[a] (μL/min/mg protein) | CL m-Heps[b] (μL/min/$10^6$ cells) | Caco-2 $P_{app}$[c] ($10^{-6}$ cm/s) | CYP1A2 $IC_{50}$ (μM) | CYP2C9 $IC_{50}$ (μM) | CYP2C19 $IC_{50}$ (μM) | CYP2D6 $IC_{50}$ (μM) | CYP3A4 $IC_{50}$ (μM) |
|---|---|---|---|---|---|---|---|
| 71 | 3 | 22 | >20 | 3.4 | >20 | 13.1 | 2.4 |

[a]Intrinsic clearance in mouse liver microsomes. [b]Intrinsic clearance in mouse hepatocytes. [c]Caco-2 cell permeability assay. $P_{app}$, permeability coefficient in the apical to basolateral direction.


https://doi.org/10.1021/acsinfecdis.1c00020
ACS Infect. Dis. 2021, 7, 1680−1689

Table 4. *In Vivo* Pharmacokinetic Profile in Male Sprague Dawley Rats (Mean ± SD; $n$ = 3 per Route) for 1

| route | dose (mg/kg) | $AUC_{0-\infty}$ (h·μM) | $F$ (%) | $C_{max}$ (μM) | $t_{max}$ (h) | $t_{1/2}$ (h) | $CL_{plasma}$ (mL/min/kg) | $V_{ss\ plasma}$ (L/kg) |
|---|---|---|---|---|---|---|---|---|
| IV | 0.9 | 4.3 ± 0.4 | - | - | - | 9.0 ± 3.1 | 7.2 ± 0.7 | 2.0 ± 0.2 |
| PO | 3 | 5.3 ± 1.6 | 37 ± 12 | 0.9 ± 0.2 | 1.5 ± 0.9 | 7.2 ± 2.4 | - | - |

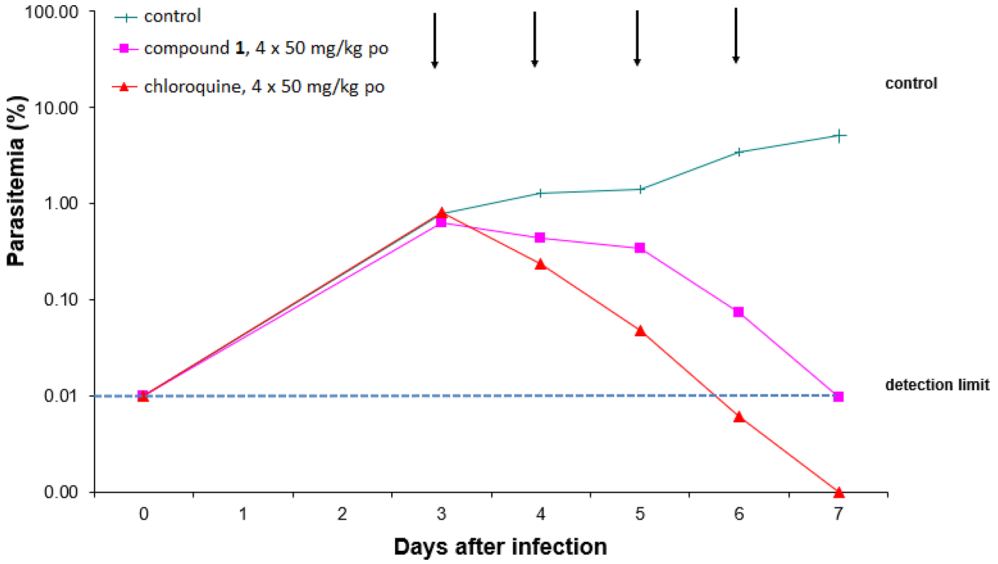



**Figure 6.** Therapeutic efficacy of **1** in the *in vivo Pf* NODscidIL2Rγnull mouse model of malaria compared with chloroquine as a reference antimalarial drug (average parasitemia in peripheral blood, $n$ = 2 mice/group). The arrows indicate the days of oral treatment at 50 mg/kg in the 4-day test.

***In Vivo* Efficacy.** Given the encouraging rat PK profile of **1**, in combination with the *in vitro* metabolic stability data in mouse, an *in vivo* proof-of-concept experiment was performed. The antimalarial efficacy of **1** was assessed in a *Pf* humanized severe combine immunodeficient (SCID) mouse model,[31] following oral administration of a 4-day treatment at 50 mg/kg given once daily. Blood concentration time profile after oral administration of 50 mg/kg of **1** is included in Figure S7. At day 7 postinfection, 99.8% reduction in parasitemia was observed in comparison to untreated control mice (Figure 6), with no adverse events. Testing the active enantiomer **1-*S*** in the same murine model confirmed *in vivo* activity with 80% parasitemia inhibition achieved at day 7 postinfection following a 4-day treatment dosage at 10 mg/kg (Table S6, Figure S8).

**Off-Target Profiling.** Finally, the risks related to possible off-targets of **1-*S*** were evaluated. Data in Table 5 illustrate the remarkable selectivity of **1-*S*** against a large 303 human kinase panel[32] obtained with 1 μM. A small-scale promiscuity panel[33] highlighted only adenosine A3, an important receptor extensively studied as a therapeutic target in cardiovascular disorder,[34] as a possible concern. This off-target activity is not surprising for an ATP-competitive inhibitor and would require further investigation through functional assays in the event this activity is not removed during the optimization process for this chemical series.

Table 5. *In Vitro* Pharmacological Profile of 1-*S*

| off-target binding assays | $IC_{50}$ (μM) |
|---|---|
| panel of 303 kinases | all >1[a] |
| ROCK1 | >10 |
| PDE4D2 | >10 |
| GR | >10 |
| PPARG | 8.4 |
| 5-HT2B | >10 |
| adenosine A3 | 1.8 |
| M1 | >10 |
| GABAA | >10 |

[a]All <18% of inhibition when tested at 1 μM.

## DISCUSSION

Although PRS is a clinically validated target, there are currently no antimalarial drugs acting through this mode of action.[18] Inhibitors binding to the ATP site of the PRS enzyme, in particular, appear attractive since they circumvent the phenotypic drug resistance associated with accumulation of L-proline, assuming ATP levels are more tightly regulated in the cell and thus resistance being less likely to occur by elevation of ATP levels. Screening of a set of *Hs*PRS ATP-site binders led to identification of 1-(pyridin-4-yl)pyrrolidin-2-one derivatives as potential novel antimalarials. Targeting the *Plasmodium* PRS, without inhibiting the human counterpart, remains a challenge with previous experience demonstrating that the repositioning process requires significant medicinal chemistry efforts.[35] Low nanomolar potency and preliminary data obtained on **1** in a human protein synthesis inhibition assay established to monitor the intracellular synthesis of artificial proline-rich protein are encouraging when considering that these molecules were initially designed for human indications. The selectivity level against the human orthologue would require further investigation in relevant biochemical assays that have been established by several groups.[13,17] Co-crystallization with *Pf* cPRS has also been carried out[13,17] and could provide useful information to advance this chemical series.


https://doi.org/10.1021/acsinfecdis.1c00020
ACS Infect. Dis. 2021, 7, 1680−1689

*In vitro* activity against *Pf* resistant strains and no cross-resistance with molecules currently in development demonstrated by compound **1** are paramount features, as pre-existing resistance would preclude development of this chemical class. Furthermore, *ex vivo* testing against clinical field isolates not only confirmed the high potency observed *in vitro* but also suggested equivalent potency against *Pf* and *Pv*. Although *Pf* is the most lethal species, *Pv* is the most widespread human malaria parasite in the Americas and Asia. Moreover, this species is responsible for most malaria relapse cases; thus, it has major public health significance.[36] The other less-represented human *Plasmodium* species *P. ovale*, *P. knowlesi*, and *P. malariae* should not be forgotten, especially in the context of mixed infections, and are planned to be assessed at the preclinical candidate stage.

The *Pf* life-cycle fingerprint studies showed dual-stage activity consistent with inhibition of cytoplasmic PRS as previously described.[9] Inducible knock-down of the *Plasmodium* gene expression was revealed to be a valuable approach for target identification.[37,38] When applied to this chemotype, this pointed to cPRS as the likely target. This was further validated by the observation of increased copy number in a region containing cPRS in resistant mutants obtained *in vitro*. The low-grade resistant mutants and increased copy number variants were obtained at a drug pressure of $3 \times IC_{50}$, which might not be optimal for such a slow killing profile. Additional experiments at higher inoculum and higher drug pressure would be required to assess fully the *in vitro* resistance profile associated with this chemotype and target.

The slow killing profile of **1** and its relative high propensity to develop resistance *in vitro*, under the conditions tested, require careful evaluation of the suitability to develop this chemical series for treatment of uncomplicated malaria. Although antimalarial drugs are intended to be used in combination therapy, which could mitigate the rapid evolution of resistance, such a slow onset of action (>48 h) would not decrease patient fever within 24 h as desired for antimalarial treatment. Fast parasite clearance would therefore need to be achieved through the partner drug. Moreover, molecules with slow onset of action need to demonstrate exceptionally long half-lives to achieve the desired parasite reduction ratio as monotherapy. This would impose an additional constraint to the combination partner since matched pharmacokinetic profiles are desirable to avoid resistance that could arise when a drug is exposed on its own to a large number of parasites. On the other hand, causal prophylaxis does not require rapid onset of action. In that regard, **1** benefits from a compelling profile with potent blood stage and antischizontal activity, the latter providing an extra 5−6 days of protection from a new infection. This is further supported by the smaller number of parasites in the liver leading to a lower anticipated risk of resistance selection[6] that would be further mitigated through combination therapy.

Ambitious goals have been set for the development of new antimalarials with the aspiration for single oral dose cure and weekly dosing for treatment of uncomplicated malaria and prophylaxis, respectively.[6] Clearance and volume of distribution are therefore two paramount PK parameters to be considered during the lead optimization process in order to achieve long half-lives. Representative compounds from this chemical series suffer from oxidative metabolism and solubility issues. Preliminary data such as metabolic identification experiments suggest reasonable scope for improvements of these aspects while maintaining potency. Despite nonoptimal metabolic stability and physicochemical properties, the frontrunner **1** exhibited acceptable PK properties in rats, which suggests the potential for delivery of a long-lasting antimalarial after optimization of this chemical series. Although the volume of distribution is acceptable (3.2 L/kg), its increase could result in extended half-life. While being aware of the potential off-target risks associated with lipophilic bases, chemical modifications such as the introduction of basic amine moieties could be considered, provided they do not have a deleterious impact on clearance. On the other hand, the observed oral *in vivo* efficacy with no associated adverse events in a murine model of human malaria clearly demonstrates the future potential of the series.

Special attention to the overall safety package is even more paramount when considering use as a prophylactic agent. Although it is debatable whether the observed toxicity previously seen with febrifugine and its analogues is related to *Hs*PRS inhibition or off-targets, PRS is being explored as an anticancer target, and its inhibition is expected to impact cell growth. As the main target population for antimalarials comprises children and pregnant women, achieving a high level of selectivity against *Hs*PRS should be the primary focus during the next steps of optimization of this chemical series. Greater than 1000-fold selectivity against *Hs*PRS was arbitrarily set as the ultimate goal for a possible preclinical candidate to minimize the risk of on-target toxicity. High selectivity against mammalian PRS (e.g., rodents, dog) is also desirable to simplify the analysis of toxicity findings during preclinical studies. Despite a reassuring profile against a large kinase panel, off-targets such as adenosine-related targets and others will be assessed as optimization progresses.

## CONCLUSIONS

We have identified a novel antimalarial scaffold exerting its dual-stage activity most likely through inhibition of the *Plasmodium* cytoplasmic PRS enzyme, a clinically validated antimalarial target. Extensive profiling of the frontrunner compound **1** and its active enantiomer **1-*S*** have revealed (i) low double-digit nanomolar potency against all malaria asexual blood stage strains tested, (ii) strong antischizontal activity, (iii) a slow killing profile, and (iv) a frequent propensity to develop moderate resistance *in vitro*. In addition, good permeability but poor aqueous solubility and moderate metabolic stability were noted for the four representative compounds of this chemical series. Metabolic identification experiments indicated the main soft spots to guide rational design of more metabolically stable analogues. Compound **1** demonstrated oral efficacy in a murine model of human malaria. Overall, 1-(pyridin-4-yl)pyrrolidin-2-one derivatives constitute a promising starting point as antimalarial PRS inhibitors but medicinal chemistry efforts are needed to optimize selectivity against *Hs*PRS while further improving antimalarial potency, pharmacokinetics, and physicochemical properties in order to achieve the profile required for new antimalarial prophylactic drugs.

## METHODS

**Chemistry.** See Supporting Information, pages S15 and S28−S36).

**Intraerythrocytic *Pf*3D7 Inhibition 72 h Growth Assay.** The intraerythrocytic *Pf*3D7 inhibition 72 h growth



https://doi.org/10.1021/acsinfecdis.1c00020
ACS Infect. Dis. 2021, 7, 1680−1689

assay involves incorporation of the DNA-intercalating dye DAPI (4′,6′,-diamidino-2-phenylindole) to monitor changes in the parasite number observed within infected erythrocytes utilizing high content confocal image analysis. This assay was performed as previously described.[22] $IC_{50}$ values were calculated through a four-parameter logistic curve fitting in GraphPad Prism v.6.

**HEK293 Viability Assay.** See Supporting Information, page S16.

**Human Cellular Protein Assay.** See Supporting Information, page S16.

***Pf* Liver Stage Assay.** See Supporting Information, pages S16−S17.

**Gamete Formation Assay.** See Supporting Information, pages S17−S18.

**Conditional Knock-Down Experiments.** See Supporting Information, page S19.

***Pf* Resistant Laboratory Strains and Cross-Resistance.** *Pf* resistant laboratory strains and cross-resistance testing was performed with the modified [$^3$H]-hypoxanthine incorporation assay as previously reported.[27] $IC_{50}$s were calculated by linear interpolation.

***Pf* and *Pv* Clinical Field Isolates.**[28] *Pf* and *Pv* clinical field isolates were collected in July 2018 from patients recruited at the Tropical Medicine Research Center − CEPEM in Porto Velho, state of Rondônia, in the Brazilian Western Amazon. The blood and the written consent were collected by a trained nurse. The study was approved by the CEPEM Ethics Committee (CAAE 61442416.7.0000.0011) (see Supporting Information pages S19−S20 for detailed protocols).

**Parasite Reduction Ratio Assay.**[29] The human biological samples were sourced ethically and their research use was in accord with the terms of the informed consents under an IRB/EC approved protocol (see Supporting Information pages S20−S21 for detailed protocols).

**MIR Studies.** MIR studies were performed on two selection groups: $10^5$ and $10^7$ parasites (see Supporting Information page S21 for detailed protocols).

**Whole-Genome Sequencing.** Whole-genome sequencing was performed on four compound **1**-resistant clones from the two selection groups (see Supporting Information page S22 for detailed protocols).

***In Vitro* DMPK and Solubility Studies.** See Supporting Information, pages S22−S25.

***In Vivo* Rat PK Studies.** *In vivo* rat PK studies were conducted using established procedures in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and the study protocols for **1** were reviewed and approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee (see Supporting Information pages S25−S26 for detailed protocols).

**SCID Mouse.** SCID mouse efficacy studies were performed as previously described[31] using *Pf*3D70087/N9 strain. The animal experiments were adhering to local and national regulations of laboratory animal welfare in Switzerland (awarded permission n°2303). Protocols are regularly reviewed and revised following approval by the local authority (Veterinäramt Basel Stadt) (see Supporting Information page S26 for detailed protocols).

**Kinase and Small Promiscuity Panels.** See Supporting Information, pages S26−S27.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.1c00020.

Figures S1−S8; Tables S1−S6; analytical data on compounds **1**, **2**, **3-S**, and **4-S**; and experimental procedures (PDF)

## ■ AUTHOR INFORMATION


### Corresponding Author

**Benoît Laleu** − *Medicines for Malaria Venture, ICC, 1215 Geneva, Switzerland*; Email: laleub@mmv.org

### Authors

**Masanori Okaniwa** − *Takeda Pharmaceutical Company Limited, Fujisawa, Kanagawa 251-8555, Japan*

**Akira Shibata** − *Takeda Pharmaceutical Company Limited, Fujisawa, Kanagawa 251-8555, Japan*

**Atsuko Ochida** − *Takeda Pharmaceutical Company Limited, Fujisawa, Kanagawa 251-8555, Japan*

**Yuichiro Akao** − *Takeda Pharmaceutical Company Limited, Fujisawa, Kanagawa 251-8555, Japan*

**Karen L. White** − *Centre for Drug Candidate Optimisation, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria 3052, Australia*

**David M. Shackleford** − *Centre for Drug Candidate Optimisation, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria 3052, Australia*

**Sandra Duffy** − *Discovery Biology, Griffith University, Nathan, Queensland 4111, Australia*

**Leonardo Lucantoni** − *Discovery Biology, Griffith University, Nathan, Queensland 4111, Australia*; orcid.org/0000-0002-7246-0677

**Sumanta Dey** − *Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, United States*

**Josefine Striepen** − *Department of Microbiology and Immunology, Columbia University Irving Medical Center, New York 10032, United States*

**Tomas Yeo** − *Department of Microbiology and Immunology, Columbia University Irving Medical Center, New York 10032, United States*

**Sachel Mok** − *Department of Microbiology and Immunology, Columbia University Irving Medical Center, New York 10032, United States*; orcid.org/0000-0002-9605-0154

**Anna Caroline C. Aguiar** − *Sao Carlos Institute of Physics, University of São Paulo, São Carlos, São Paulo 13563-120, Brazil*

**Angelika Sturm** − *TropIQ Health Sciences, 6534 AT Nijmegen, The Netherlands*

**Benigno Crespo** − *Global Health, GlaxoSmithKline R&D, Madrid 28760, Spain*

**Laura M. Sanz** − *Global Health, GlaxoSmithKline R&D, Madrid 28760, Spain*

**Alisje Churchyard** − *Department of Life Sciences, Imperial College London, London SW7 2AZ, United Kingdom*

**Jake Baum** − *Department of Life Sciences, Imperial College London, London SW7 2AZ, United Kingdom*

**Dhelio B. Pereira** − *Tropical Medicine Research Center of Rondonia, Porto Velho, Rondonia 76812-329, Brazil*

**Rafael V. C. Guido** − *Sao Carlos Institute of Physics, University of São Paulo, São Carlos, São Paulo 13563-120, Brazil*
**Koen J. Dechering** − *TropIQ Health Sciences, 6534 AT Nijmegen, The Netherlands*
**Sergio Wittlin** − *Swiss Tropical and Public Health Institute, 4002 Basel, Switzerland; University of Basel, 4002 Basel, Switzerland*
**Anne-Catrin Uhlemann** − *Division of Infectious Diseases, Department of Medicine, Columbia University Irving Medical Center, New York 10032, United States*
**David A. Fidock** − *Department of Microbiology and Immunology and Division of Infectious Diseases, Department of Medicine, Columbia University Irving Medical Center, New York 10032, United States*
**Jacquin C. Niles** − *Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, United States;* orcid.org/0000-0002-6250-8796
**Vicky M. Avery** − *Discovery Biology, Griffith University, Nathan, Queensland 4111, Australia*
**Susan A. Charman** − *Centre for Drug Candidate Optimisation, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria 3052, Australia*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsinfecdis.1c00020

### Author Contributions

B.L.: project leader, writing, original draft; B.L., A.Sh., M.O.: conceptualization (repurposing project); A.Sh., M.O: compound design; V.M.A., S.A.C.: supervisors at Griffith and CDCO respectively; A.O., Y.A., K.L.W., S.Du., L.L.: project team members; V.M.A., S.Du., L.L.: 3D7/HEK assays; S.A.C., D.M.S., K.L.W.: DMPK; J.C.N., S.De.: conditional knockdown; J.S., T.Y., S.M., A.C.U., D.A.F.: resistance selection studies, whole-genome sequencing; ACCA, DBP, RVCG: field isolates; A.St., K.J.D.: *Pf* liver; B.C., L.M.S.: PRR; A.C., J.B.: DGFA; S.W.: cross-resistance, SCID. Edits and contributions from all authors who read and approved the final manuscript.

### Funding

Research reported in this publication was supported by the Global Health Innovative Technology (GHIT) Fund under the project title "New Hit-to-Lead Activity for New Antimalarials between MMV and Takeda" (Project ID: H2016-205). Gamete formation experiments were funded through MMV support, project RD-08−2800 (to J.B., A.C.). J.B. is supported by an Investigator Award from Wellcome (100993/B/13/Z). Clinical field isolates experiments were funded through MMV support, project RD-16-1066 (to R.V.C.G., A.C.C.A.). R.V.C.G. is supported by Sao Paulo Research Foundation (FAPESP - CEPID grant 2013/07600-3). D.A.F. gratefully acknowledges support from MMV. S.M. is a recipient of a Human Frontiers Science Program Long-Term Fellowship (LT000976/2016-L). J.C.N. is supported by Bill and Melinda Gates Foundation grants (OPP1132312 and OPP1162467).

### Notes

The authors declare the following competing financial interest(s): B.L. is a MMV employee; M.O., A.O., Y.A. are Takeda employees; B.C., L.M.S. are GSK employees.

## ■ ACKNOWLEDGMENTS

Chemistry design, protein synthesis, off-target binding, and kinase panel assays have been performed as in-kind contribution by Takeda. We thank TCGLifesciences and Dr. Abhijit Kundu for chemistry support. The authors wish to thank the Australian Red Cross Blood Service for the provision of human red blood cells in accordance with agreement 19-05QLD-21. Dr. Takeshi Yura (Jubilant Biosys) is thanked for mentoring this project. Dr. Yusuke Tominari, Dr. Masato Yoshida, Dr. Takaharu Hirayama, Dr. Shinichi Imamura, Dr. Mitsuyuki Shimada, Dr. Takashi Ichikawa, Dr. Izumi Nomura, Dr. Masahiro Kamaura, Dr. Brice Campo are thanked for facilitating project transfer from Takeda to MMV.

## ■ REFERENCES

(1) World Health Organization (WHO) (2020) *World Malaria Report 2020*, WHO, Geneva.
(2) The malERA Refresh Consultative Panel on Insecticide and Drug Resistance (2017) malERA: An updated research agenda for insecticide and drug resistance in malaria elimination and eradication. *PLoS Med. 14*, e1002450.
(3) Nsanzabana, C. (2019) Resistance to artemisinin combination therapies (ACTs): do not forget the partner drug! *Trop. Med. Infect. Dis. 4*, E26.
(4) Wells, T. N. C., Van Huijsduijnen, R. H., and Van Voorhis, W. C. (2015) Malaria medicines: a glass half-full? *Nat. Rev. Drug Discovery 14*, 24−42.
(5) Tse, E. G., Korsik, M., and Todd, M. H. (2019) The past, present and future of anti-malarial medicines. *Malar. J. 18*, 93.
(6) Burrows, J. N., Duparc, S., Gutteridge, W. E., van Huijsduijnen, R. H., Kaszubska, W., Macintyre, F., Mazzuri, S., Möhrle, J. J., and Wells, T. N. C. (2017) New Developments in AntiMalarial Target Candidate and Product Profiles. *Malar. J. 16*, 26.
(7) Coatney, G. R., Cooper, W. C., Culwell, W. B., White, W. C., and Imboden, C. A., Jr. (1950) Studies in human malaria. XXV. Trial of febrifugine, an alkaloid obtained from *Dichroa febrifuga* Lour. against the Chesson strain of *Plasmodium vivax*. *J. Natl. Malar. Soc. 9*, 183−186.
(8) Chaudhuri, R. N., Dutta, B. N., and Chakravarty, R. N. (1954) *Dichroa febrifuga* and a synthetic hydrangea alkaloid in malaria. *Indian Medical Gazette 89*, 660−666.
(9) Herman, J. D., Pepper, L. R., Cortese, J. F., Estiu, G., Galinsky, K., Zuzarte-Luis, V., Derbyshire, E. R., Ribacke, U., Lukens, A. K., Santos, S. A., Patel, V., Clish, C. B., Sullivan, W. J., Jr., Zhou, H., Bopp, S. E., Schimmel, P., Lindquist, S., Clardy, J., Mota, M. M., Keller, T. L., Whitman, M., Wiest, O., Wirth, D. F., and Mazitschek, R. (2015) The cytoplasmic prolyl-tRNA synthetase of the malaria parasite is a dual-stage target of febrifugine and its analogs. *Sci. Transl. Med. 7*, 288ra77.
(10) Pines, M., and Spector, I. (2015) Halofuginone - the multifaceted molecule. *Molecules 20*, 573−594.
(11) Jiang, S., Zeng, Q., Gettayacamin, M., Tungtaeng, A., Wannaying, S., Lim, A., Hansukjariya, P., Okunji, C. O., Zhu, S., and Fang, D. (2005) Antimalarial activities and therapeutic properties of febrifugine analogs. *Antimicrob. Agents Chemother. 49*, 1169−1176.
(12) Herman, J. D., Rice, D. P., Ribacke, U., Silterra, J., Deik, A. A., Moss, E. L., Broadbent, K. M., Neafsey, D. E., Desai, M. M., Clish, C. B., Mazitschek, R., and Wirth, D. F. (2014) A genomic and evolutionary approach reveals non-genetic drug resistance in malaria. *Genome Biol. 15*, 511.
(13) Hewitt, S. N., Dranow, D. M., Horst, B. G., Abendroth, J. A., Forte, B., Hallyburton, I., Jansen, C., Baragaña, B., Choi, R., Rivas, K. L., Hulverson, M. A., Dumais, M., Edwards, T. E., Lorimer, D. D., Fairlamb, A. H., Gray, D. W., Read, K. D., Lehane, A. M., Kirk, K., Myler, P. J., Wernimont, A., Walpole, C., Stacy, R., Barrett, L. K., Gilbert, I. H., and Van Voorhis, W. C. (2017) Biochemical and

structural characterization of selective allosteric inhibitors of the *Plasmodium falciparum* drug target, prolyl-tRNA-synthetase. *ACS Infect. Dis. 3*, 34−44.
(14) Adachi, R., Okada, K., Skene, R., Ogawa, K., Miwa, M., Tsuchinaga, K., Ohkubo, S., Henta, T., and Kawamoto, T. (2017) Discovery of a novel prolyl-tRNA synthetase inhibitor and elucidation of its binding mode to the ATP site in complex with l-proline. *Biochem. Biophys. Res. Commun. 488*, 393−399.
(15) Arita, T., Morimoto, M., Yamamoto, Y., Miyashita, H., Kitazawa, S., Hirayama, T., Sakamoto, S., Miyamoto, K., Adachi, R., Iwatani, M., and Hara, T. (2017) Prolyl-tRNA synthetase inhibition promotes cell death in SK-MEL-2 cells through GCN2-ATF4 pathway activation. *Biochem. Biophys. Res. Commun. 488*, 648−654.
(16) Shibata, A., Kuno, M., Adachi, R., Sato, Y., Hattori, H., Matsuda, A., Okuzono, Y., Igaki, K., Tominari, Y., Takagi, T., Yabuki, M., and Okaniwa, M. (2017) Discovery and pharmacological characterization of a new class of prolyl-tRNA synthetase inhibitor for anti-fibrosis therapy. *PLoS One 12*, e0186587.
(17) Jain, V., Yogavel, M., Oshima, Y., Kikuchi, H., Touquet, B., Hakimi, M. A., and Sharma, A. (2015) Structure of prolyl-tRNA synthetase-halofuginone complex provides basis for development of drugs against malaria and toxoplasmosis. *Structure 23*, 819−829.
(18) Nyamai, D. W., and Bishop, O. T. (2020) Identification of selective novel hits against *Plasmodium falciparum* prolyl tRNA synthetase active site and a predicted allosteric site using in silico approaches. *Int. J. Mol. Sci. 21*, 3803.
(19) Wirth, D. F., Mazitschek, R., Santos, S. A., Tye, M. A., and Payne, N. C. (2019) Inhibitors of prolyl-tRNA-synthetase. PCT Int. Appl. WO2019237125A1.
(20) Hirayama, T., Hirata, Y., Tominari, Y., Iwamura, N., Sasaki, Y., Asano, M., Takagi, T., Okaniwa, M., Yoshida, M., Ichikawa, T., and Imamura, S. (2018) Preparation of heterocyclic amide compounds as PRS inhibitors. PCT Int. Appl. WO2018052065A1.
(21) Hirayama, T., Hirata, Y., Tominari, Y., Iwamura, N., Sasaki, Y., Asano, M., Takagi, T., Okaniwa, M., Yoshida, M., and Imamura, S. (2018) Preparation of heterocyclic amide compounds as PRS inhibitors. PCT Int. Appl. WO2018052066A1.
(22) Duffy, S., and Avery, V. M. (2012) Development and Optimization of a Novel 384-Well Anti-Malarial Imaging Assay Validated for High-Throughput Screening. *Am. J. Trop. Med. Hyg. 86*, 84−92.
(23) Boes, A., Spiegel, H., Kastilan, R., Bethke, S., Voepel, N., Chudobová, I., Bolscher, J. M., Dechering, K. J., Fendel, R., Buyel, J. F., Reimann, A., Schillberg, S., and Fischer, R. (2016) Analysis of the dose-dependent stage-specific in vitro efficacy of a multi-stage malaria vaccine candidate cocktail. *Malar. J. 15*, 279.
(24) Delves, M. J., Straschil, U., Ruecker, A., Miguel-Blanco, C., Marques, S., Dufour, A. C., Baum, J., and Sinden, R. E. (2016) Routine *in vivo* culture of *P. falciparum* gametocytes to evaluate novel transmission-blocking interventions. *Nat. Protoc. 11*, 1668−1680.
(25) Khan, S. (2016) Recent advances in the biology and drug targeting of malaria parasite aminoacyl-tRNA synthetases. *Malar. J. 15*, 203.
(26) Ganesan, S. M., Falla, A., Goldfless, S. J., Nasamu, A. S., and Niles, J. C. (2016) Synthetic RNA-protein modules integrated with native translation mechanisms to control gene expression in malaria parasites. *Nat. Commun. 7*, 10727.
(27) Snyder, C., Chollet, J., Santo-Tomas, J., Scheurer, C., and Wittlin, S. (2007) *In Vitro* and *in vivo* interaction of synthetic peroxide RBx11160 (OZ277) with piperaquine in *Plasmodium* models. *Exp. Parasitol. 115*, 296−300.
(28) Aguiar, A. C. C., Pereira, D. B., Amaral, N. S., De Marco, L., and Krettli, A. U. (2014) *Plasmodium vivax* and *Plasmodium falciparum ex vivo* susceptibility to anti-malarials and gene characterization in Rondônia, West Amazon, Brazil. *Malar. J. 13*, 73.
(29) Sanz, L. M., Crespo, B., De-Cozar, C., Ding, X. C., Llergo, J. L., Burrows, J. N., Garcia-Bustos, J. F., and Gamo, F. J. (2012) *P. falciparum* in vitro killing rates allow to discriminate between different antimalarial mode-of-action. *PLoS One 7*, e30949.
(30) Coteron, J. M., Marco, M., Esquivias, J., Deng, X., White, K. L., White, J., Koltun, M., El Mazouni, F., Kokkonda, S., Katneni, K., Bhamidipati, R., Shackleford, D. M., Angulo-Barturen, I., Ferrer, S. B., Jimenez-Diaz, M. B., Gamo, F. J., Goldsmith, E. J., Charman, W. N., Bathurst, I., Floyd, D., Matthews, D., Burrows, J. N., Rathod, P. K., Charman, S. A., and Phillips, M. A. (2011) Structure-guided lead optimization of triazolopyrimidine-ring substituents identifies potent *Plasmodium falciparum* dihydroorotate dehydrogenase inhibitors with clinical candidate potential. *J. Med. Chem. 54*, 5540−5561.
(31) Jimenez-Díaz, M. B., Mulet, V., Viera, S., Gomez, V., Garuti, H., Ibanez, J., Alvarez-Doval, A., Shultz, L. D., Martínez, A., Gargallo-Viola, D., and Angulo-Barturen, I. (2009) Improved murine model of malaria using *Plasmodium falciparum* competent strains and non-myelodepleted NOD-scid IL2Rgammanull mice engrafted with human erythrocytes. *Antimicrob. Agents Chemother. 53*, 4533−4536.
(32) Hirozane, Y., Toyofuku, M., Yogo, T., Tanaka, Y., Sameshima, T., Miyahisa, I., and Yoshikawa, M. (2019) Structure-based rational design of staurosporine-based fluorescent probe with broad-ranging kinase affinity for kinase panel application. *Bioorg. Med. Chem. Lett. 29*, 126641.
(33) Sameshima, T., Yukawa, T., Hirozane, Y., Yoshikawa, M., Katoh, T., Hara, H., Yogo, T., Miyahisa, I., Okuda, T., Miyamoto, M., and Naven, R. (2020) Small-scale panel comprising diverse gene family targets to evaluate compound promiscuity. *Chem. Res. Toxicol. 33*, 154−161.
(34) Nishat, S., Khan, L. A., Ansari, Z. M., and Basir, S. F. (2016) Adenosine A3 receptor: a promising therapeutic target in cardiovascular disease. *Curr. Cardiol. Rev. 12*, 18−26.
(35) Lotharius, J., Gamo-Benito, F. J., Angulo-Barturen, I., Clark, J., Connelly, M., Ferrer-Bazaga, S., Parkinson, T., Viswanath, P., Bandodkar, B., Rautela, N., Bharath, S., Duffy, S., Avery, V. M., Möhrle, J. J., Guy, R. K., and Wells, T. (2014) Repositioning: the fast track to new anti-malarial medicines? *Malar. J. 13*, 143.
(36) Battle, K. E., Gething, P. W., Elyazar, I. R., Moyes, C. L., Sinka, M. E., Howes, R. E., Guerra, C. A., Price, R. N., Baird, K. J., and Hay, S. I. (2012) The global public health significance of *Plasmodium vivax*. *Adv. Parasitol. 80*, 1−111.
(37) Nasamu, A. S., Glushakova, S., Russo, I., Vaupel, B., Oksman, A., Kim, A. S., Fremont, D. H., Tolia, N., Beck, J. R., Meyers, M. J., Niles, J. C., Zimmerberg, J., and Goldberg, D. E. (2017) Plasmepsins IX and X are essential and druggable mediators of malaria parasite egress and invasion. *Science 358*, 518−522.
(38) Istvan, E. S., Das, S., Bhatnagar, S., Beck, J. R., Owen, E., Llinas, M., Ganesan, S. M., Niles, J. C., Winzeler, E., Vaidya, A. B., and Goldberg, D. E. (2019) *Plasmodium* Niemann-Pick type C1-related protein is a druggable target required for parasite membrane homeostasis. *eLife 8*, e40529.


https://doi.org/10.1021/acsinfecdis.1c00020
*ACS Infect. Dis.* 2021, 7, 1680−1689