High-Throughput Screening Platform To Identify Inhibitors of Protein Synthesis with Potential for the Treatment of Malaria

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ABSTRACT Artemisinin-based combination therapies have been crucial in driving down the global burden of malaria, the world’s largest parasitic killer. However, their efficacy is now threatened by the emergence of resistance in Southeast Asia and sub-Saharan Africa. Thus, there is a pressing need to develop new antimalarials with diverse mechanisms of action. One area of Plasmodium metabolism that has recently proven rich in exploitable antimalarial targets is protein synthesis, with a compound targeting elongation factor 2 now in clinical development and inhibitors of several aminoacyl-tRNA synthetases in lead optimization. Given the promise of these components of translation as viable drug targets, we rationalized that an assay containing all functional components of translation would be a valuable tool for antimalarial screening and drug discovery. Here, we report the development and validation of an assay platform that enables specific inhibitors of Plasmodium falciparum translation (PfIVT) to be identified. The primary assay in this platform monitors the translation of a luciferase reporter in a P. falciparum lysate-based expression system. Hits identified in this primary assay are assessed in a counter-screen assay that enables false positives that directly interfere with the luciferase to be triaged. The remaining hit compounds are then assessed in an equivalent human IVT assay. This platform of assays was used to screen MMV’s Pandemic and Pathogen Box libraries, identifying several selective inhibitors of protein synthesis. We believe this new high-throughput screening platform has the potential to greatly expedite the discovery of antimalarials that act via this highly desirable mechanism of action.

KEYWORDS Plasmodium, malaria, in vitro translation, protein synthesis, drug discovery, antimalarials

Malaria is a life-threatening disease that results in more than 400,000 deaths every year, many of which occur among children under the age of 5 years (1). The disease results from infection with unicellular, protozoan parasites from the genus Plasmodium, with the vast majority of deaths caused by Plasmodium falciparum and P. vivax. Current front-line therapies for malaria are under constant threat from the emergence of drug resistance (2, 3). The current standard of care for the treatment of malaria, recommended by the World Health Organization (WHO), is reliant upon artemisinin-based combination therapies (ACTs) (4). However, clinical artemisinin resistance is now prevalent across Southeast Asia (5) and sub-Saharan Africa (6), threatening these combination therapies. Thus, there is a pressing need for new and effective drugs to provide chemoprotection, prevent transmission, and treat (vivax) relapse.

The development of new drugs capable of treating this devastating parasitic disease has been confounded by a number of factors. The life cycle of the Plasmodium parasite is
extremely complex. Following initial transmission via the bite of the *Anopheles* mosquito, sporozoites infect the hepatocytes of the host liver. Parasites replicate and differentiate within hepatocytes prior to entering the bloodstream, where merozoites infect red blood cells. Intraerythrocytic infection is characterized by a rapid expansion of the parasite population (schizogony). At this stage, some parasites differentiate into sexual forms (gametocytes) that can be taken up through the bite of a mosquito and transmitted to other humans. Long-term control of malaria will likely require the development of multiple compounds that demonstrate activity against multiple parasite life cycle stages, extremely challenging in terms of drug discovery. In addition, antimalarial drug discovery has been hindered by the general lack of robustly validated drug targets in *Plasmodium*, severely limiting target-focused screening programs.

One area of *Plasmodium* metabolism that has proven relatively rich in exploitable antimalarial targets is protein synthesis. Doxycycline is widely used for malaria chemoprophylaxis and assumed to act through inhibition of parasite protein synthesis via binding to the 30S ribosomal subunit. The fungal secondary metabolite cladosporin, a potent inhibitor of *Plasmodium* growth in blood and liver stages, specifically targets lysyl-tRNA synthetase (LysRS) (7). Optimization of a chromone hit identified in a biochemical screen led to the first *P. falciparum* LysRS (*Pf* LysRS) inhibitor with efficacy in a malaria mouse model (8). In addition to LysRS, a series of novel bicyclic azetidines demonstrating in vivo efficacy were found to specifically target cytosolic phenylalanyl-tRNA synthetase (PheRS) (9), while the potent antimalarials borrelidin and halofuginone inhibit threonyl-tRNA synthetase (ThrRS) (10) and prolyl-tRNA synthetase (ProRS) (11), respectively. Aminoacyl-tRNA synthetases catalyze aminoacylation of tRNAs with their cognate amino acids. *P. falciparum* translation elongation factor 2 (*Pf* eEF2), responsible for the GTP-dependent translocation of the ribosome along mRNA, has also been identified as a promising target (12). Indeed, M5717, a compound specifically targeting *PfeEF2*, is now undergoing first-in-human trials (13).

The advantage of antimalarials that target core, essential biological processes, such as protein synthesis, is that they have the potential to be effective against multiple life cycle stages of *Plasmodium*. Strategies that can facilitate the identification of protein synthesis inhibitors with diverse modes of action are highly desirable. Previous studies have reported the development of cell-free or in vitro translation (IVT) assays capable of identifying antimalarials that target protein synthesis (14–16). Their use has enabled the screening of small libraries as well as validation of the drug mechanism of action (MoA) with respect to protein translation (15). However, these assays have not yet proven suitable or scalable to support high-throughput screening of larger compound libraries. Here, we describe the development of an assay platform devoted to the identification of specific inhibitors of *P. falciparum* translation. This platform is comprised of a *P. falciparum* lysate-based IVT assay that monitors the translation of a luciferase reporter, a counter-screen assay to identify false positives that interfere directly with the luciferase reporter, and an equivalent human IVT assay that is used to guide the identification of selective inhibitors of parasite translation. These 384-well plate assays are capable of identifying a diverse range of translation-specific inhibitors. To demonstrate the power and utility of this platform, pilot screens of the Medicines for Malaria Venture (MMV) open-access Pathogen Box and Pandemic Box compound libraries were carried out, leading to the identification of several novel inhibitors of *P. falciparum* protein synthesis.

**RESULTS AND DISCUSSION**

Optimization and validation of high-throughput *PfIVT* and *HsIVT* assays. Our *P. falciparum* in vitro translation (*PfIVT*) assay is built upon previously established assays (14, 17) and is summarized in Fig. 1A. In this assay, successful reconstitution of *Plasmodium* protein translation is reported via synthesis of a luciferase reporter, a counter-screen assay to identify false positives that interfere directly with the luciferase reporter, and an equivalent human IVT assay that is used to guide the identification of selective inhibitors of parasite translation. These 384-well plate assays are capable of identifying a diverse range of translation-specific inhibitors. To demonstrate the power and utility of this platform, pilot screens of the Medicines for Malaria Venture (MMV) open-access Pathogen Box and Pandemic Box compound libraries were carried out, leading to the identification of several novel inhibitors of *P. falciparum* protein synthesis.
introduced upstream of the reporter gene, and the 3’ UTR from \( P. falciparum \) histidine-rich protein 2 (\( PfHrp2 \)) gene (19) was introduced downstream. For the reporter construct to support our complementary human IVT assay (\( HsIVT \)), the 5’ UTR was replaced with an internal ribosome entry site (IRES) from encephalomyocarditis virus (EMCV) (20) and the 3’ UTR was replaced with a poly(A) tail (21). It is worth noting that the 5’ UTR of EMCV was unable to initiate translation in the \( PfIVT \) assay and vice versa. Finally, a 30-bp poly(A) tail, present within the vector and downstream of the luciferase gene, was inserted to enhance mRNA stability (21).

Translationally active \( P. falciparum \) or human embryonic kidney (HEK) 293F lysates were supplemented with accessory, helper, amino acid, and energy regeneration solutions (see Materials and Methods for details). In vitro translation reactions were initiated by the addition of purified mRNA to each reaction well. Using this basic assay format, a number of parameters were assessed and optimized, namely, assay temperature, time, mRNA concentration, and reaction volume (summarized in Fig. S2 in the supplemental material). In addition, we compared the luminescence signal of the CBG firefly luciferase with CBG99, derived from the click beetle \( Photinus pyralis \) (18). Direct comparison of these two luciferases revealed that the luminescence signal for CBG99 was over 2-fold higher than for the original firefly luciferase (Fig. S2), leading us to base our reporter constructs around this superior luciferase. Based on these preliminary studies, the optimal assay parameters were established as reaction volume of 5 \( \mu L \), assay temperature of 32°C, mRNA concentration of 1,000 ng/\( \mu L \), and reaction time of 210 min. Under these conditions, the assay reported a signal-to-background ratio of at least 10 and robust \( Z' \) values above 0.5 in a 384-well plate format.

A selection of established inhibitors of protein translation in \( Plasmodium \) was then used to validate the \( PfIVT \) assay, including cycloheximide (inhibitor of the translocation step in the elongation), emetine (inhibitor of 80S ribosome), borrelidin (inhibitor of ThrRS), halofuginone (inhibitor of ProRS), cladosporin (inhibitor of LysRS), and an analogue of DDD107498/M5717 (inhibitor of eEF2), now in clinical trials for the treatment of malaria (Fig. 2). In addition, an inhibitor that mimics a transition state analogue of LysRS (DDD01712277) was also assessed. These compounds, known to inhibit various aspects of cytosolic protein translation in \( Plasmodium \), were successfully identified by
this assay, validating its ability to identify different aspects of in vitro translation were selected to validate our IVT assay. (A) Cycloheximide is an inhibitor of the translocation step in elongation (46). (B) Borrelidin is an inhibitor of ThrRS (47). (C) DDD00197451 is an inhibitor of translation via eEF2 (44). (D) Halofuginone is an inhibitor of ProRS (11). (E) DDDD001712277 is an inhibitor of the 80S ribosome (43). (G) Cladosporin is an inhibitor of LysRS (7). All curves shown are from a single technical replicate and are representative of data for at least two biological replicates. IC_{50} values (insets) are weighted means ± SD from at least two biological replicates.
Initially, compounds were screened in single point (30 μM) against the PfIVT assay. Analysis of our single-point screens revealed normal distributions of inhibition (for the Pathogen Box, the median inhibition $\bar{x}$ was 1.00% and the standard deviation $s$ was 23.61% [Fig. 3A]; for the Pandemic Box, $\bar{x}$ was 2.08% and $s$ was 19.75% [Fig. 3B]). DDD00197451, an analogue of eEF2 inhibitor M5717, was added as an internal control in all plates and inhibited 97% translation ($Pf$IVT), comparable to the positive control cycloheximide. Interestingly, both libraries presented nitazoxanide (MMV688991) among the primary hits, supporting the robustness of PfIVT assay in identifying hits independently. Using a cutoff of 2.5$\sigma$ (98.7% confidence assuming a normal hit distribution), PfIVT assay identified hit rates of 4.5% for the Pathogen Box (18 compounds demonstrating $\geq$58.02% inhibition) and 3.5% for the Pandemic Box (14 compounds demonstrating $\geq$47.29% inhibition). These represent unusually high hit rates compared to standard target-based screens but were consistent with hit rates seen for high-content and cell-based screens (24). Our interpretation is that since translation is a complex, multicomponent process offering multiple potential targets, the PfIVT assay aligns more closely with phenotype-based screens. It should also be noted that the Pathogen Box contains a number of compounds confirmed to inhibit $P. falciparum$ growth in vitro (25), which could have positively influenced the hit rate seen in this study.

Primary hits identified in the single-point screen were reassessed in dose-dependent PfIVT assays to confirm their activity and accurately measure their potency. Six primary hits from the Pathogen Box (out of 18) and 4 from the Pandemic Box (out of 14) presented IC$_{50}$ values of <1 μM, with the most potent (nitazoxanide [MMV688991]) returning an IC$_{50}$ value of 20 nM (Table 1). To confirm hits as bona fide inhibitors of in vitro translation, rather
**TABLE 1** Collated PFIVT and counterscreen data for compounds from MMV's Pathogen Box demonstrating >50% inhibition in a single-point IVT screen at 30 μM$^a$

| Compound         | PFIVT inhibition at 30 μM (%) | IC$_{50}$ value (μM) | Counterscreen |
|------------------|-------------------------------|----------------------|---------------|
|                  | PFIVT                         | HsIVT                |               |
| MMV688407        | 98.7                          | 6 ± 0.09             | 5.2 ± 0.02    | >30 |
| MMV667494        | 97.7                          | 0.3 ± 0.09           | >30           | >30 |
| MMV688991        | 97.0                          | 0.02 ± 0.008         | 0.04 ± 0.0003 | 0.02 ± 0.008 |
| MMV688547        | 96.7                          | 1.8 ± 0.07           | 2.7 ± 0.02    | >30 |
| MMV687807        | 95.1                          | 0.09 ± 0.007         | 0.5 ± 0.006   | 25 ± 0.8 |
| MMV688362        | 93.5                          | 2.8 ± 0.08           | 5.6 ± 0.04    | >30 |
| MMV634140        | 88.4                          | 2.9 ± 0.09           | >30           | >30 |
| MMV019189        | 84.4                          | 0.03 ± 0.009         | 7.7 ± 0.3     | 7 ± 0.6 (≥30) |
| MMV67243         | 82.5                          | 2.2 ± 0.08           | 13 ± 0.2      | 0.9 ± 0.09 |
| MMV637953        | 78.7                          | 3.1 ± 0.07           | >30           | >30 |
| MMV688474        | 78.4                          | 16 ± 0.9             | 14 ± 0.07     | >30 |
| MMV676008        | 74.9                          | 0.4 ± 0.08           | 12 ± 0.4      | 21 ± 0.6 |
| MMV67730         | 72.4                          | 3.9 ± 0.8            | >30           | >30 |
| MMV675998        | 68.9                          | 13 ± 0.9             | 18 ± 0.13     | >30 |
| MMV688271        | 65.7                          | 18 ± 0.9             | 13 ± 0.16     | >30 |
| MMV676350        | 65.1                          | 16 ± 0.8             | 6.7 ± 0.13    | >30 |
| MMV687188        | 64.9                          | 2.4 ± 0.07 (≥30)     | >30           | >30 |
| MMV688372        | 62.9                          | 0.6 ± 0.06           | >30           | 11 ± 0.8 (≥30) |
| MMV676512        | 54.2                          | >30                  | 22 ± 0.46     | >30 |

$^a$All IC$_{50}$ values represent the weighted means ± standard deviations of two technical replicates. Compounds with IC$_{50}$ values 1 order of magnitude (10-fold) higher for the counterscreen than for the PFIVT assay are considered viable hits and are shaded.

than false positives interfering with the CBG99 luciferase reporter, compounds were next assessed against our validated luciferase counterscreen. False positives were identified as compounds presenting IC$_{50}$ values <10-fold more potent in the PFIVT assay than in the counterscreen. Using this criterion, two compounds from the Pathogen Box were excluded as false positives: the most potent hit, MMV688991/nitazoxanide, a broad-spectrum antiviral (26, 27), as well as MMV687243. Thus, 16 compounds from the Pathogen Box were confirmed as inhibitors of PFIVT, with 5 compounds demonstrating submicromolar potency (Table 1). Remarkably, 10 of the 12 hit compounds from the Pandemic Box demonstrated similar IC$_{50}$ values in the PFIVT and the counterscreen (Table 2), indicating that these are inhibitors of the CBG99 luciferase rather than translation. The remaining two confirmed PFIVT inhibitors (MMV1578897 and MMV1580839) both demonstrated submicromolar potency, with MMV1578897 particularly active (IC$_{50}$ value, 40 nM).

The 18 confirmed inhibitors of *P. falciparum* translation were assessed in HsIVT assays to detect potential liabilities as inhibitors of human translation. As expected, MMV667494, an analogue of the PfEEF2 inhibitor M5717, was inactive against HsIVT (IC$_{50}$ value, >30 μM), in keeping with compounds from this series previously demonstrating a high degree of selective inhibition for *P. falciparum* growth compared to human cells (12). In contrast, the selectivity index for compound MMV687807 inhibiting PFIVT (IC$_{50}$, 0.09 μM) compared to HsIVT (IC$_{50}$, 0.5 μM) was ≥5-fold, earmarking this compound as potentially a generic inhibitor of translation. Indeed, the established ribosome inhibitor emetine (HsIVT IC$_{50}$, 1.4 μM; PFIVT IC$_{50}$, 0.35 μM) demonstrated a similarly narrow selectivity window. Combined, these data demonstrate the importance of our HsIVT assay to prioritize primary hits with the potential to specifically inhibit parasite translation.

**Prioritization and assessment of hits.** A total of 7 compounds were identified as attractive primary hits targeting *P. falciparum* translation. Of these 7, MMV019189 (PFIVT IC$_{50}$, 0.03 μM; HsIVT IC$_{50}$, 7.7 μM), MMV676008 (PFIVT IC$_{50}$, 0.4 μM; HsIVT IC$_{50}$, 12 μM), MMV667244 (PFIVT IC$_{50}$, 0.3 μM; HsIVT IC$_{50}$, >30 μM), and MMV688372 (PFIVT IC$_{50}$, 0.6 μM; HsIVT IC$_{50}$, >30 μM) demonstrated promising potency against parasite translation, with selectivity windows ≥30-fold over inhibition of translation in human lysate (summarized in Table 1). The remaining 3 compounds—MMV634140 (PFIVT IC$_{50}$,
TABLE 2 Collated PfIVT and counterscreen data for compounds from MMV’s Pandemic Box demonstrating >50% inhibition in a single-point IVT screen at 30 μM

| Compound          | PfIVT inhibition at 30 μM (%) | IC50 value (μM) | Counterscreen |
|-------------------|-------------------------------|-----------------|---------------|
|                   | PfIVT                         | HsIVT           |               |
| MMV688991         | 98.3                          | 0.02 ± 0.007    | 0.04 ± 0.0003 | 0.04 ± 0.008 |
| MMV002459         | 87.3                          | 29 ± 0.9 (>30)  | 22 ± 0.3      | >30          |
| MMV1578897        | 93.4                          | 0.04 ± 0.008    | 0.18 ± 0.001  | 0.4 ± 0.08   |
| MMV1582495        | 89.9                          | 0.6 ± 0.07      | 3.1 ± 0.04    | 1.7 ± 0.08   |
| MMV1578578        | 74.6                          | 3.7 ± 0.07      | 10 ± 0.12     | 1.3 ± 0.08   |
| MMV1579781        | 72.4                          | 8.1 ± 0.09      | >30           | 0.5 ± 0.08   |
| MMV1580839        | 73.3                          | 0.8 ± 0.08      | 7.6 ± 0.12    | 8.3 ± 0.08   |
| MMV124656         | 72.6                          | 14 ± 0.9        | 17 ± 0.16     | 3.8 ± 0.08   |
| MMV108465         | 62.6                          | 17 ± 0.8        | 23 ± 0.11     | 7.6 ± 0.5    |
| MMV247764         | 63.2                          | 14 ± 0.7        | 23 ± 0.2      | 3.2 ± 0.06   |
| MMV141011         | 51.8                          | >30             | >30           | 10 ± 0.8     |
| MMV003291         | 52.9                          | 2 ± 0.07        | >30           | 0.5 ± 0.08   |
| MMV1634391        | 52.4                          | 17 ± 0.6        | 1.1 ± 0.01    | >30          |

All IC50 values represent the weighted mean ± standard deviation of two technical replicates. Compounds with IC50 values 1 order of magnitude (10-fold) higher for the counterscreen compared to the PfIVT assay are considered viable hits and are shaded.

2.9 μM; HsIVT IC50 (>0 μM), MMV1578897 (PfIVT IC50 0.04 μM; HsIVT IC50 0.18 μM), and MMV1580839 (PfIVT IC50 0.8 μM; HsIVT IC50 8 μM)—also showed significant selectivity in the PfIVT assay versus the HsIVT, albeit with more modest safety windows.

We next assessed the potency of the prioritized compounds against asexual-blood-stage (ABS) *P. falciparum* (Table 3). With the exception of MMV676008, all 7 of these compounds demonstrated some level of activity against ABS parasites. Three compounds demonstrated submicromolar activity. MMV667494 and MMV634140, both analogues of the Pfef2 inhibitor M5717/DDD00107498, were the most potent in ABS assay, with 50% effective concentration (EC50) values of 10 and 41 nM, respectively. MMV019189 also demonstrated promising activity in the PfIVT assay versus the HsIVT, albeit with more modest safety windows.

*Pf* 7
TABLE 3 Structures and antimalarial data for compounds demonstrating sub μM activity in the PfIVT assay

| Compound (reference) | Structure | P. falciparum asexual-blood-stage EC_{50} (μM) | PfIVT IC_{50} (μM) |
|----------------------|-----------|---------------------------------------------|-------------------|
| MMV019189 (35)       | ![](image1) | 0.75<sup>a</sup>                            | 0.03 ± 0.009      |
| MMV1578897 (30)      | ![](image2) | 5<sup>b</sup>                               | 0.04 ± 0.008      |
| MMV687807 (29)       | ![](image3) | 1.8<sup>a</sup>                             | 0.09 ± 0.007      |
| MMV667494 (44)       | ![](image4) | 0.010<sup>a</sup>                           | 0.3 ± 0.09        |
| MMV676008 (48)       | ![](image5) | >20<sup>a</sup>                             | 0.4 ± 0.08        |
| MMV688372 (31)       | ![](image6) | 13.6                                        | 0.6 ± 0.06        |
| MMV634140 (12)       | ![](image7) | 0.09<sup>a</sup>                            | 3 ± 0.09          |

(Continued on next page)
potency against *P. falciparum* (EC$_{50}$ 14 μM), though not enough to be prioritized. Interestingly, this compound also shares structural features with two established proteasome inhibitors currently in clinical development for the treatment of visceral leishmaniasis (32, 33). MMV1580839 (PfIVT IC$_{50}$ 900 nM), reported as having an EC$_{50}$ value of 5 μM against *P. falciparum*, is described as an inhibitor of the bacterial methyltransferases ErmC and ErmAM, blocking the ability of these enzymes to methylate rRNA (34). Finally, the most active confirmed hit in our PfIVT assay, MMV019189 (IC$_{50}$ 30 nM), is a previously reported antimalarial with nanomolar activity against multiple developmental stages of the parasite (35). Undoubtedly, additional work will be required to further define the molecular targets of MMV019189 and other actives described here.

One strategy could be to carry out thermal protein profiling (TPP) with compounds using the enriched lysates prepared for our PfIVT assay (36, 37). TPP takes advantage of the fact that binding of a ligand to its target can thermally stabilize the target. This approach enables the thermal stability of all proteins within a lysate to be monitored and compared in the presence and absence of test compounds, thus enabling potential targets to be identified. For compounds where structure-activity relationships are better understood, linkers could be attached and used as handles to facilitate the pulldown of targets from IVT lysates and establish molecular targets. Alternatively, parasites resistant to test compounds can be generated through *in vitro* evolution. Whole-genome analysis of resistant clones can lead to the identification of genomic changes pointing to genes encoding compound targets (38). Our ultimate goal will be to structurally enable drug discovery programs focused on evolving more potent or selective analogues of MMV019189 and other promising hit compounds.

**Conclusions.** In summary, the combination of our IVT assay platform with *in vitro* potency data has led to the identification of one priority hit (MMV019189) and several other compounds of interest that are associated with inhibition of protein synthesis in *P. falciparum* for the first time. These studies clearly demonstrate the power of this assay platform to generate robust data that can be used to prioritize compounds acting via this highly desirable mechanism of action.

**MATERIALS AND METHODS**

**Generation of reporter constructs to support *P. falciparum* and human IVT assays.** To support PfIVT assays, the production of luciferase mRNA was required. The previously described plasmid pHLH-1, which comprises the 5′ UTR of the *P. falciparum* histidine-rich protein 3 (PfHrp3) gene upstream of a firefly luciferase reporter gene and the 3′ UTR from the histidine-rich protein 2 (PfHrp2) gene downstream (19), was used as a starting point. This plasmid was modified to introduce a T7 bacteriophage terminator sequence using site-directed mutagenesis. Briefly, Phusion DNA polymerase (New England BioLabs [NEB]) was used in PCRs in conjunction with a forward (FW; CGC GCT TGG CGA ATC ATG GTC A) and reverse (RV; GCT AGT TAT TGC TCA GCG GCA ATT AAC CCT CAC TAA AGG GAA CAA AAG) primers under the following conditions: 1× cycle of denaturation at 98°C for 30 s followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 58°C for 15 s, and extension at 72°C for 120 s. The resulting vector (modified pHLH) was linearized with HindIII (NEB) and used in PCRs in conjunction with a forward (FW; CGC GCT TGG CGA ATC ATG GTC A) and reverse (RV; GCT AGT TAT TGC TCA GCG GCA ATT AAC CCT CAC TAA AGG GAA CAA AAG) primers under the following conditions: 1× cycle of denaturation at 98°C for 30 s followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 58°C for 15 s, and extension at 72°C for 120 s. The resulting vector (modified pHLL) was linearized with HindIII (NEB), and the PCR-amplified cbg99 luciferase gene was inserted using Gibson Assembly (NEB). The cbg99 gene (Fig. S1) was PCR amplified from Promega’s pCBG vector using the Phusion DNA polymerase (NEB) and the following primers: FW, ATA TTA ATA CAG TTA TTT TAA AAA AAT GGT GAA GCG TGA GAA AAA TG, and RV, TTT TAA TCT ATT ATT AAA TAA GCT TCT AAC CGC CCG CC. The resulting plasmid (modified pHLL-CBG99) was used in the production of mRNA for the *P. falciparum* IVT assay.

To support the HisIVT assay, the pT7CFE vector (Thermo Fisher Scientific), containing an IRES from
the encephalomyocarditis virus (EMCV) and a 30-bp poly(A) region, and was digested with MseI and XhoI (NEB). The cbg99 gene, PCR amplified from the pCBG vector using FW primer GAA AAA CAC GAT GAT AAT ATG GCC ACC ATG GTG ACG CTT GAG AAA AAT G and RV primer CAG TGG TGG TGG TGC TGC TCG AGA CCG CCG GC, encompassing homology to pT7CFE. The PCR product was then cloned into the digested pT7CFE vector using Gibson Assembly (NEB). The resulting plasmid (pT7CFE-CBG99) was used in the production of mRNA for the human IVT assay.

**Assay validation construct.** In preliminary assay development experiments, a plasmid that facilitated expression of a hemagglutinin (HA)-tagged version of the firefly luciferase (Fig. S1) was used. In this study, the modified pHllH-1 plasmid, described above, was used as a starting point. The plasmid was digested with NsiI and HindIII (NEB) to release the Cbg luciferase. A Genestring (Life Technologies) containing homology to the digested plasmid was cloned using Gibson Assembly inserting a 3×HA tag and tobacco etch virus (TEV) cleavage site into the vector (Fig. S1). This formed a universal vector (pHLHUNI) that could be used to insert genes of interest fused to an N- or C-terminal 3×HA tag and TEV cleavage site. The newly formed vector was linearized with HindIII and the firefly luciferase (lux) gene inserted, again using Gibson Assembly.

**Western blotting.** Proteins were separated on 12% SDS-PAGE gels and then transferred to nitrocellulose membranes using an iBlot 2 (Thermo Fisher) system as per the manufacturer’s instructions. The membrane was blocked in 10% (wt/vol) milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-T) for 1 h. Following blocking, the membrane was incubated in 2% (wt/vol) milk in PBS-T containing the HA tag-specific primary antibody 12CA5 (Cell Signaling) at a dilution of 1:1,000 for 1 h. The membrane was then washed with PBS-T (three times for 5 min). Following a washing, membranes were incubated in 2% milk in PBS-T containing an anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP; Merck) at a dilution of 1:5,000 for 1 h prior to washing in PBS-T (three times for 5 min). HA-tagged protein on the membrane was detected using the ECL Western blot detection reagent (GE Healthcare) as per the manufacturer’s instructions and visualized using a Gel Doc XR+ imaging system (Bio-Rad).

**In vitro transcription of mRNA.** Reactions to produce cbg99 mRNA were run using 40 mM HEPES (pH 7.4), 18 mM magnesium acetate, ribonucleotide triphosphates (5 mM each), 2 mM spermidine, 40 mM dithiothreitol (DTT), 0.0025 U/μL inorganic pyrophosphatase, modified pHllH-CBG99 for the parasite assay and pT7CFE-CBG99 for the human assay (70 ng/μL), RNase inhibitor (3 U/μL), and T7 RNA polymerase (10 U/μL). This reaction mixture was incubated for 100 min at 37°C, and DNase I (0.1 mg/mL) was added for the final 20 min of the incubation. The reaction mixture was then diluted 1:1 with RNase-free H₂O and RNA was precipitated using 1.6 M (lulose membranes using an iBlot 2 (Thermo Fisher) system as per the manufacturer’s instructions and visualized using a Gel Doc XR+ imaging system (Bio-Rad).

**Parasite strain and culture conditions.** The Plasmodium falciparum reference strain 3D7 used throughout this study was cultured as previously described (39). Briefly, cultures incubated at 37°C in a humidified atmosphere of 1% O₂ and 3% CO₂ in balance with N₂ were maintained in RPMI 1640 media supplemented with 5% human red blood cells (provided by the Scottish National Blood Transfusion Service), 25 mM HEPES, 2 mM L-glutamine, 0.5% AlbuMAX II (Gibco), 12 mM sodium bicarbonate, 0.2 mM hypoxanthine, and 20 mg/L gentamicin (pH 7.3).

**P. falciparum lysate preparation.** 3D7 parasites were synchronized following two rounds of treatment with o-sorbitol (5%), as previously described (40). The hematocrit was reduced from 5% (for standard culture) to 1.5 to 2% and cultures were transferred into HYPERflasks (Corning). Fresh medium was added once or twice daily. Late trophozoite/schizont stage parasites were harvested by centrifugation (1,800 × g, 15 min, 4°C, low brake) once parasitemia reached 8 to 15%. Harvested red blood cells (infected) were lysed by incubation in 0.1% (wt/vol) saponin on ice for 10 min with gentle agitation. Free parasites were harvested by centrifugation (2,800 × g, 10 min, 4°C) and washed 3 times in wash buffer (WB; 100 mM potassium acetate, 2.5 mM magnesium acetate, 45 mM HEPES [pH 7.4], 250 mM sucrose, 2 mM dithiothreitol, and 15 μM leupeptin) to remove lysed red blood cell debris. The resulting pellet was resuspended in 1 volume of WB supplemented with Complete EDTA-free protease inhibitor cocktail (Roche; 1 tablet/20 mL) and human RNase A inhibitor (Sigma; 5 U/mL). Parasite lysis was achieved by nitrogen cavitation using a prechilled 45-mL Parr cell disruption vessel (1,500 lb/in², 60 min, 4°C). To clarify the lysate, it was centrifuged (10,000 × g, 15 min, 4°C), and the supernatant was collected, transferred into fresh tubes, and centrifuged (30,000 × g, 15 min, 4°C). As a quality assurance step for each lysate, the RNA content of the resulting supernatant was determined using a NanoDrop spectrophotometer (Shimadzu). Supernatants with RNA levels of >250 ng/μL were aliquoted (100 μL), flash frozen in liquid nitrogen, and stored at −80°C.

**HEK 293F cell culture.** FreeStyle human embryonic kidney (HEK) 293F cells (Thermo Fisher Scientific) were grown in 500-mL polycarbonate Erlenmeyer vented flasks (Corning) containing FreeStyle 293 expression medium (Life Technologies) upon reaching a density of approximately 2 × 10⁶. Cells were then centrifuged at 1,000 × g for 10 min at 4°C, the medium was discarded, and the resulting pellet was washed once in buffer II containing 20 U of human placental RNase inhibitor (Sigma-Aldrich) and Complete EDTA-free inhibitor cocktail (Roche) before being centrifuged at 2,800 × g for 10 min. Clarified HEK cell lysate was obtained in the same way as described for P. falciparum lysate.
**P. falciparum** and human IVT assays. PRVT and HsIVT reactions (5 μL final volume) were performed in 384-well plates (Corning) containing 50% lysate from cells (either *P. falciparum* or HEK 293F), 10% amino acid solution (final concentration of 400 μM for each amino acid in 60 mM KOH; biotrackabiot), 10% energy recovery solution (final assay concentrations of 40 mM HEPES [pH 7.4], 1.5 mM ATP, 0.15 mM GTP, 40 μM creatine phosphate, and 40 μM creatine phosphokinase), 10% helper solution (final assay concentrations of 200 μM cysteine, 2% polyethylene glycol 3000 [PEG 3000], 1 mM spermidine, 0.5 mM folic acid, and 15 μM leupeptin), 10% supplemental salt solution (22.5 mM HEPES [pH 7.4], 50 mM potassium acetate, 1 mM magnesium acetate, 1 mM DTT, 1 μM human placental RNase inhibitor, 0.1 mM leupeptin), and 10% *cbg99* luciferase mRNA (3,000 ng/mL). A master mix solution containing all components was prepared on ice prior to assays, and 5 μL was dispensed into wells using an automated Integra VIAFLO 16-channel 12.5-μL pipette. Assays were incubated for 210 min at 32°C, and luciferase buffer (5 μL; final assay concentrations of 45 mM HEPES [pH 7.4], 1 mM MgCl₂, 1 mM ATP, 5 mM DTT, 1% Triton X-100, 10 mg/mL bovine serum albumin [BSA], 1 mg/mL o-luciferin, and 1 × Pierce firefly signal enhancer) was added to read luminescence using a BMG PheraStar plate reader. Cycloheximide was used as a positive control in both PRVT and HsIVT reactions, representing 100% inhibition. Hits were assessed as inhibitors of translation in both IVT assays in 8-point dose-dependent assays ranging from 30 μM to 13.7 nM (1:3 dilutions). Relative activity of luciferase was calculated based on positive-control reactions.

**False-positive counterscreen.** Hits identified in our primary assay screen were assessed for their potential to interfere with the *cbg99* luciferase reporter. For this counterscreen, the master mix containing all the components required for translation (as described above) was incubated for 210 min at 32°C, resulting in the in vitro translation of *cbg99* luciferase. The produced luciferase was then incubated with test compounds for 5 min prior to the addition of luciferase reaction buffer. Luciferase I inhibitor (Calbiochem) was used at 50 μM as a positive control in each assay, representing 100% inhibition. Hits from the primary screen were assessed as luciferase inhibitors in 8-point dose-dependent assays ranging from 30 μM to 13.7 nM (1:3 dilutions). Levels of luciferase inhibition were determined relative to the positive control.

**Inhibitor studies.** Test compounds (10 mM in 100% dimethyl sulfoxide [DMSO]) were dispensed into 384-well assay plates using the acoustic Echo 550 dispenser (Labcyte). Compound libraries were screened at a single inhibitor concentration (30 μM), and compounds inhibiting >50% PRVT activity were selected for dose-dependent analysis. For dose-response assays, compound concentrations ranging from 30 μM to 13.7 nM (1:3 dilutions) were assessed in 8-point inhibition assays. IC₅₀ values were determined using XLFit using a 4-parameter equation. Cycloheximide (50 μM) was used as a positive control for PRVT on every assay plate representing 100% inhibition.

**Compounds and libraries.** Established inhibitors of IVT—cycloheximide (41), boremiladin (42), halofuginone (11), and emetine (43)—were purchased from Sigma. Open-access compound libraries Pathogen Box and the Pandemic Response Box, both 400-compound libraries, were kindly provided by Medicines for Malaria Venture (MMV). DDD00197451 (44) and DDD01712277 (12) were synthesized as previously described. Cladosporin was kindly provided by Chris Walpole from the Structural Genomics Consortium.

**Quality control of library compounds.** Hits identified in our primary assay screen were subjected to mass spectrometry to confirm purity and compound identity. For this, library compounds in DMSO were diluted 20-fold in a clean, u-shaped, deep-well 384-well plate. The plate was heat sealed (Waters heat sealer) and vortexed. Samples were then injected into an ultrahigh-performance liquid chromatography (UHPLC)-mass spectrometry (MS) 2020 single-quadrupole mass spectrometer with atmospheric pressure chemical ionization and electrospray ionization (ESI) probes (DG-20A3R and DG-20A5R degasser; 2 × LC-30 AD binary pumps; SIL-30AC MP multiplate autosampler with sample chiller; CTO-20AC plus 2 column switching valve; SPD-M30A UV/visible diode array detector with 1-cm flow cell). Library compounds (2 μL) were injected into a Hypersil Gold column (Thermo Fisher Scientific; 1.9-μm internal diameter, 50-μm length, 175-Å pore size) and HPLC separated using a gradient of solvent A (water/0.05% formic acid) and solvent B (acetonitrile/0.05% formic acid) at 0.6 mL/min and 50°C. Detection was performed at 254 nm (40°C) using a mass spectral range from 50 to 1,000 Da (both positive and negative modes; scan speed, 15,000 Da/s; interface voltage, 4.5 kV). Shimadzu Labsystems 5.91 software was used to control sample injections into the LC-MS system and to analyze processed data (integration of peak areas and assessment of masses). Trazadone hydrochloride and reserpine were used as internal standards.

**SYBR green-based *P. falciparum* growth inhibition assay.** Our SYBR green assay growth assay was based on a previous report (45). In brief, 96-well black clear-bottom plates (Corning) were preprinted with a compound and normalized with DMSO to 0.5% of a total assay volume of 100 μL. Highly synchronized ring-stage parasites and blood were added to be a final parasitemia and 1% hematocrit. The compounds were incubated with parasites for 72 h prior to freezing at −20°C (to aid cell lysis). The plate was thawed on ice and lysis buffer (20 mM Tris pH 7.5, 5 mM EDTA, 0.008% [w/v] saponin, 0.08% [v/v]) containing SYBR green I (Thermo Fisher Scientific) at a final concentration of 0.02% (v/v) was added. The 96-well plate was equilibrated to room temperature for 1 h before fluorescence was determined on a TECAN Infinite Pro 200 microplate reader using green fluorescent protein (GFP) filters (excitation, 485 nm; emission, 535 nm). Data were fitted to a two-parameter equation using GraFit version 7.0 (Erichugas Software), and EC₅₀ values were calculated. An excess of the standard inhibitor mefloquine (10 μM) was used to define 0% parasite growth.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.**
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