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EXPERIMENTAL THERAPEUTICS

Novel Transmission-Blocking Antimalarials Identified by High-Throughput Screening of *Plasmodium berghei* Ookluc

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ABSTRACT Safe and effective malaria transmission-blocking chemotherapeutics would allow a community-level approach to malaria control and eradication efforts by targeting the mosquito sexual stage of the parasite life cycle. However, only a single drug, primaquine, is currently approved for use in reducing transmission, and drug toxicity limits its widespread implementation. To address this limitation in antimalarial chemotherapeutics, we used a recently developed transgenic *Plasmodium berghei* line, Ookluc, to perform a series of high-throughput *in vitro* screens for compounds that inhibit parasite fertilization, the initial step of parasite development within the mosquito. Screens of antimalarial compounds, approved drug collections, and drug-like molecule libraries identified 185 compounds that inhibit parasite maturation to the zygote form. Seven compounds were further characterized to block gametocyte activation or to be cytotoxic to formed zygotes. These were further validated in mosquito membrane-feeding assays using *Plasmodium falciparum* and *P. vivax*. This work demonstrates that high-throughput screens using the Ookluc line can identify compounds that are active against the two most relevant human *Plasmodium* species and provides a list of compounds that can be explored for the development of new antimalarials to block transmission.

KEYWORDS malaria, transmission blocking, compound screen

Malaria is endemic in 85 countries, and in 2020, there were 241 million cases of the disease, resulting in 627,000 deaths (1). The *Plasmodium* parasite, the causative agent of malaria, is transmitted to humans through the bite of infected *Anopheles* mosquitoes, which take up the circulating mature gametocyte forms of the parasite when they ingest blood from an infected individual. Once ingested by a mosquito, male and female gametocytes rapidly form gametes in the mosquito midgut and undergo fertilization, forming a zygote, which matures after 18 to 24 h into the ookinete form (2). The ookinete invades the midgut epithelium to the basal lamina and subsequently develops into an oocyst (2), wherein sporozoites form. After around 15 days, the sporozoites migrate to the salivary glands of the mosquito (3), where they can then be inoculated into another individual during a subsequent mosquito blood meal, thereby initiating a new infection.

Despite the continuous decreases in malaria incident cases and mortality between 2000 and 2015, progress has plateaued over the past 7 years (1). Vector control, through the use of insecticides and bed nets, has been critical for malaria control programs. However, parasite transmission seems to have reached a point of resilience, with only moderate responses to the intensification of the available control measures. To make matters worse, the perpetuation of low transmission aiming at elimination is fragile,

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and malaria cases often increase in situations of a national or global crisis, as in the case of the coronavirus disease 2019 (COVID-19) pandemic (4), which underpins the consensus that new tools to block malaria transmission are needed.

The WHO global technical strategy for malaria 2016–2030 (http://www.who.int/malaria/en/) considers that an important addition to the toolkit for malaria control will be the implementation of transmission-blocking (TB) chemotherapies. Among the currently available antimalarials, primaquine is the only one indicated for use specifically for TB intervention in some regions (5), and recent studies support the indication of tafenoquine for TB intervention (6, 7). However, primaquine and tafenoquine are contraindicated in patients with glucose-6-phosphate dehydrogenase (G6PD) enzyme deficiencies due to the risk of hemolytic anemia (8). Thus, developing new, safe, and widely available TB antimalarials is critical for malaria control and elimination efforts.

In this context, we have recently used the mouse parasite *Plasmodium berghei* to develop a line that expresses a recombinant nanoluciferase (nLuc) reporter only when zygotes are formed, named Ookluc (9). The engineered nLuc reporter parasite Ookluc can serve to assess gamete viability, fertilization, and zygote maturation *in vitro*, in formats amenable to high-throughput (HT) compound screening. Here, we present the application of this assay for the identification of novel TB compounds.

RESULTS

High-throughput screens. To increase the capacity of screens with Ookluc, the assay was optimized for 1,536-well plate screening. The primary screening assays in the 1,536-well format had an average Z-score of 0.68 (range, 0.41 to 0.86). A total of 6,631 compounds from three libraries were tested: the Mechanism of Interrogation PlatE (MIPE) library, with 2,476 small molecules with annotated mechanisms of action (10–12); the NCATS Pharmaceutical Collection (NPC), with 2,816 compounds approved by either U.S., European Union, Canada, or Japan regulatory agencies (13, 14); and the novel NCATS Malaria Active Compounds (NMAC), with 1,339 compounds effective against asexual-stage *Plasmodium falciparum* parasites with IC_{50} (compound concentration that inhibits 50% of normalized parasite proliferation) values of $<2\ \mu$M in the original primary screen. The NPC and MIPE libraries consist of approved drugs and characterized preclinical compounds, respectively, that target primarily eukaryotic pathways, whereas the NMAC collection contains drugs, characterized compounds, and novel chemotypes with asexual antimalarial activity.

Screening of the three libraries was performed in a quantitative high-throughput manner with full concentration responses assessed (15): 321 unique compounds demonstrating high-quality concentration-response curve (CCv2) values with IC_{50}s of $<2\ \mu$M were identified. These compounds along with other select compounds based on known antimalarial activity or relatedness to potent conversion assay inhibitors were selected for screening as a validation set (total of 346 compounds) (Fig. 1). The compound validation set was replated from a stock solution, screened in three independent parasite conversion assays, and counterscreened against the nLuc reporter (for reporter interference) and HepG2 cells to assess mammalian cell toxicity. Of the 346 compounds, 220 compounds were validated with high-quality concentration-response curves (CCv2, -1.1, -1.2) in the conversion assay with IC_{50}s of $<2\ \mu$M. Of these validated potent compounds, 35 compounds demonstrated some inhibition of the nLuc recombinant enzyme with activity at $<10\ \mu$M (with a curve class value of -1.1, -1.2, or -2.2), or HepG2 cell toxicity at $<10\ \mu$M (same curve class values). Excluding these counterscreened compounds, 185 selective, potent compounds were identified and validated in the Ookluc *in vitro* assay with IC_{50}s of $<2\ \mu$M (Fig. 1; screening data are presented in Tables S1 to S6 in the supplemental material). These compounds were also evaluated against the *P. falciparum* Dd2 asexual stage to assess stage-specific or multistage activities; 37 of the compounds were >10-fold more potent in the conversion assay with transgenic *P. berghei*, and 39 were >10-fold more potent against the asexual stages of *P. falciparum* Dd2 parasites, with the remaining 111 compounds being in between (Table S7). Importantly, the

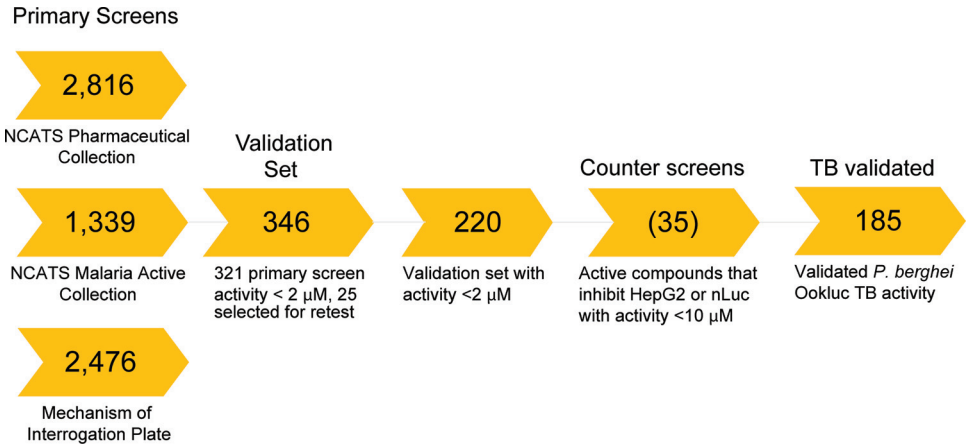

FIG 1 Illustration of the high-throughput screens with Ookluc. Three compound collections were used for the primary screen, from which 346 compounds, including 321 compounds with an IC_{50} of <2 μM, were selected for the validation screens. IC_{50} values of <2 μM were confirmed for 220 compounds, 35 of which had inhibitory activity against nLuc or were toxic to HepG2 cells, leaving 185 compounds with specific inhibitory activity at <2 μM against Ookluc fertilization.

higher potency of some compounds in the conversion assay is due to the resistance of *P. falciparum* Dd2, for example, the case of pyrimethamine. In other cases, the comparison may point to differences in metabolic pathways. From the validated compounds, seven were selected for further studies (Table 1 and Fig. S1). These were prioritized to evaluate compounds with various efficacies against the asexual stage, in addition to the Ookluc activity, and diverse suspected modes of action.

Characterization of TB activity. The seven compounds were initially screened in exflagellation assays to evaluate their ability to block *P. berghei* fertilization (Fig. 2A). DDD107498, fascaplysin, hesperadin, and nanchangmycin blocked the formation of exflagellation centers by activated gametocytes, indicating that they impede fertilization by inhibiting male gamete formation. In contrast, pyronaridine, batimastat, and quinacrine reduced but did not abolish the formation of exflagellation centers (Fig. 2A), while their ability to block ookinete formation was confirmed (Fig. 2B), showing that they may have activity either against female gametocytes (not assessed) or, downstream of gametocyte activation, against gamete fertilization or zygote maturation.

The formation of zygotes in the presence of pyronaridine, batimastat, and quinacrine was tested 1 or 6 h after gametocyte-to-gamete conversion (Fig. 3A and B). Normal numbers of zygotes were observed in assay mixtures treated with pyronaridine 1 h after activation, while the numbers of zygotes were drastically reduced in assay mixtures treated with batimastat and were abolished with quinacrine (Fig. 3A), suggesting that pyronaridine has minimal activity against gametocytes and gametes, while it remains possible that batimastat and quinacrine block zygote formation through the inhibition of female

TABLE 1 Activities of the seven compounds selected for further characterization against Ookluc and *P. falciparum* Dd2 asexual stages

	IC_{50} (μM)	
Compound	**Ookluc**	***P. falciparum* Dd2 asexual stages**[a]
DDD107498	0.001	0.014
Fascaplysin	0.022	NA
Pyronaridine	1.399	0.026
Hesperadin	0.437	0.528
Batimastat	0.415	0.011
Quinacrine	1.313	0.167
Nanchangmycin	0.329	0.013

[a]NA, not applicable.

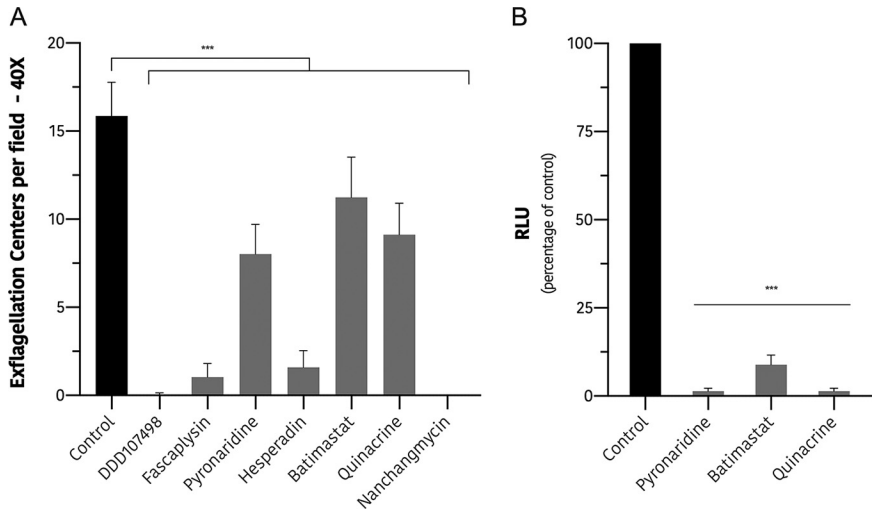

FIG 2 Inhibition of gametocyte activation. (A) Number of exflagellation centers per 40× field counted by light microscopy (means and standard deviations [SD]) after 15 min of gametocyte activation in ookinete medium in the presence of 10 μM each indicated compound. Results are representative of data from three independent assays. Bars are the means and SD. Ordinary analysis of variance (ANOVA) with Dunnett's test was used for statistics. ***, $P < 0.001$. (B) Zygote formation in 6-h conversion assays (means and SD) in the presence of 10 μM each indicated compound, measured by relative light units (RLU) relative to the control (percentage). Results are representative of data from eight independent assays. Bars are the means and SD. Ordinary ANOVA with Dunnett's test was used for statistics. ***, $P < 0.001$; *, $P < 0.05$.

gametocytes/gametes. After 6 h, zygotes were absent in all conversion assays (Fig. 3B), an indication that pyronaridine blocks ookinete formation through a cytotoxic effect on zygotes.

***P. falciparum* SMFAs and *P. vivax* DMFAs.** To test whether compounds identified through Ookluc HT screening indeed have transmission-blocking activity, the seven selected compounds were tested in mosquito membrane-feeding assays (MFAs) with *P. falciparum* or *Plasmodium vivax*.

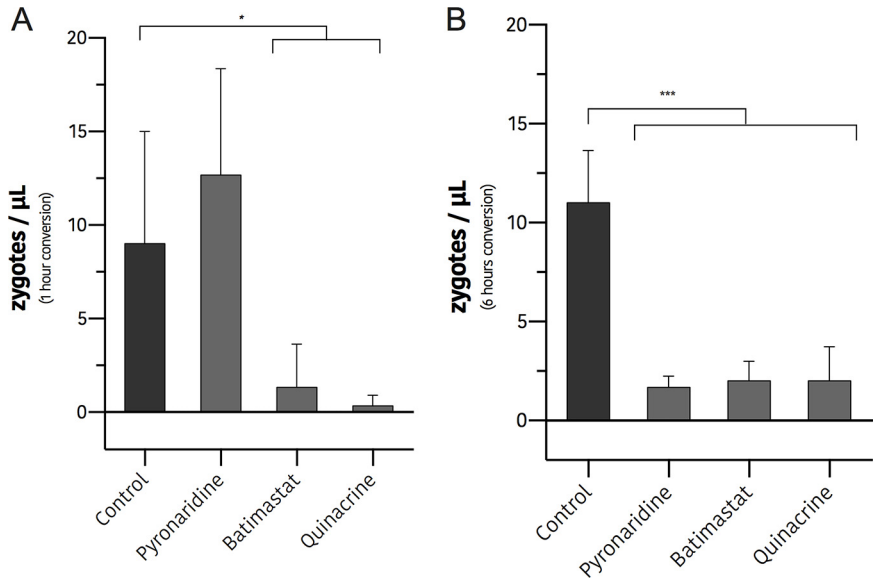

FIG 3 Inhibition of zygote formation. Shown are the numbers of zygotes per microliter counted by light microscopy ($n = 3$) in 1 μL after 1 h (A) or 6 h (B) of gametocyte activation in ookinete medium in the presence of 10 μM each indicated compound. Bars are the means and SD. Results are representative of data from three independent assays. Ordinary ANOVA with Dunnett's test was used for statistics. ***, $P < 0.001$; *, $P < 0.05$.

TABLE 2 Inhibition of *P. falciparum* oocyst formation by the seven compounds selected for further characterization, added to gametocytes 2 min before *A. stephensi* feeding[a]

Compound	Drug concn (μM)	No. of mosquitoes infected/total no. of mosquitoes	Estimated % inhibition of oocyst counts (95% CI [low, high])	*P* value
DDD107498	10	12/40	93.1 (87.3, 96.7)	0.001
	2	23/40	80.1 (65.8, 89.3)	0.001
	0.4	22/40	78.8 (63.5, 88.5)	0.001
Fascaplysin	10	2/40	99.7 (99.0, 99.9)	0.001
	2	25/40	74.0 (54.7, 86.0)	0.001
	0.4	24/40	82.0 (68.6, 90.1)	0.001
Pyronaridine	10	12/40	97.2 (94.6, 98.9)	0.001
	2	20/40	63.1 (36.1, 79.0)	0.001
	0.4	26/40	51.9 (15.1, 71.7)	0.01
Hesperadin	10	0/20	100.0 (99.5, 100.0)	0.001
	2	0/20	100.0 (99.1, 100.0)	0.001
	0.4	0/40	99.9 (99.3, 99.9)	0.001
	0.08	5/20	92.1 (82.1, 97.3)	0.001
	0.016	11/20	43.7 (−22.5, 75.3)	0.147
Batimastat	10	20/20	16.4 (−84.8, 62.0)	0.641
Quinacrine	10	18/20	52.1 (−3.4, 78.8)	0.061
Nanchangmycin	10	0/20	100.0 (99.7, 100.0)	0.001

[a]Data are from *P. falciparum* SMFAs with compounds added to parasites 2 min before mosquito feeding. Note that each compound at each concentration was tested in either a single assay (when the total mosquito number was 20) or two independent assays (when the total mosquito number was 40). CI, confidence interval.

Screening the human parasite *P. falciparum* using the standard membrane-feeding assays (SMFAs), only batimastat and quinacrine failed to demonstrate TB activity (Table 2 and Table S8), highlighting the predictive screening potential of the Ookluc reporter line in identifying compounds with TB activity against human *Plasmodium* parasites. Hesperadin showed strong inhibitory activity at concentrations of as low as 80 nM (Table 2). In *P. vivax* direct membrane-feeding assays (DMFAs), batimastat, pyronaridine, fascaplysin, and quinacrine did not exhibit significant TB activity, while hesperadin, DDD107498, and nanchangmycin showed strong inhibitory activities comparable to those observed in the *P. falciparum* SMFAs (Table 3 and Table S9).

The *P. falciparum* and *P. vivax* MFAs were performed by adding the compounds to infected blood only 2 min before mosquito feeding, so any measured effect can be attributed exclusively to compound activity against the mosquito stages. Because 400 nM pyronaridine added 2 min before feeding reduced *P. falciparum* oocyst formation 51.9%, and because pyronaridine is a licensed and implemented antimalarial, it was also tested at lower concentrations that reflected plasma levels after dosing (16) in *P. falciparum* SMFAs by adding the drug 24 or 48 h before feeding. While 50 nM pyronaridine did not inhibit oocyst formation, incubation of the gametocytes for 48 h with 400 nM drug demonstrated oocyst inhibition of 66.7% (Table 4 and Table S8), comparable to that with no preincubation at the same concentration of 400 nM (51.9% inhibition) (Table 2).

DISCUSSION

In this study, we applied the *P. berghei* line Ookluc (9), a luciferase reporter line, for HT screening of compound libraries in a 1,536-well plate format. The HT assay was used to evaluate 6,631 compounds against fertilization, identifying 185 compounds with TB potential. Many identified compounds have predicted modes of action related to DNA replication and tubulin polymerization/depolymerization, likely inhibiting the

TABLE 3 Inhibition of *P. vivax* oocyst formation by the seven compounds selected for further characterization, added to gametocytes 2 min before *A. darlingi* feeding[a]

Compound	Drug concn (μM)	No. of mosquitoes infected/total no. of mosquitoes	Estimated % inhibition of oocyst counts (95% CI [low, high])	*P* value
DDD107498	10	1/60	99.9 (99.7, 100.0)	0.001
	2	1/40	100.0 (99.8, 100.0)	0.001
	0.4	41/60	86.5 (76.1, 93.1)	0.008
Fascaplysin	10	52/60	−30.0 (−130.2, 23.6)	0.347
Pyronaridine	10	57/60	−17.6 (−102.1, 31.0)	0.583
Hesperadin	10	27/60	99.4 (98.9, 99.7)	0.001
	2	33/60	95.0 (91.3, 97.2)	0.001
	0.4	35/60	77.0 (60.2, 87.7)	0.001
Batimastat	10	58/60	−109.6 (−253.4, −24.1)	0.006
Quinacrine	10	59/60	39.3 (−5.3, 64.7)	0.073
Nanchangmycin	10	0/40	100.0 (99.7, 100.0)	0.001

[a]Data are from *P. vivax* MFAs with compounds added to parasites 2 min before mosquito feeding. Note that each compound at each concentration was tested in either two (when the total mosquito number was 40) or three (when the total mosquito number was 60) independent assays.

three mitotic events for male gametogenesis. Some potent compounds identified in our screen have previously been identified as having gametocytocidal activity, like Torin-2, which was recently shown to target *P. falciparum* phosphatidylinositol 4-kinase (17, 18). Seven compounds were selected for further evaluation and were tested in mosquito infections with *P. falciparum* or *P. vivax*.

Recent HT screens to identify TB compounds, including those with dual-stage activity targeting both asexual and TB stages, have been performed (18–20), yielding highly potent hits. However, in our methodology, compounds were added immediately prior to gametocyte activation *in vitro* or in mosquito feeding assays, limiting the drug exposure of the gametocyte forms and, thus, focusing the assay on the early parasite sexual stages that are formed in the mosquito midgut. This narrow stage of the malaria life cycle was chosen as it involves profound changes in metabolic activity that may be specifically targeted by chemotherapeutics (21). Our previous work demonstrated that compound activity against asexual stages or gametocytes does not predict activity against parasite early stages in the mosquito (9). Therefore, we screened 3 different compound collections, NMAC to identify molecules with dual-stage activity and NPC and MIPE to identify molecules with antimalarial activity yet possibly not active against asexual stages or gametocytes.

The 185 compounds identified as having strong activity against *Plasmodium* fertilization have different putative modes of action, reflecting the abundance of metabolic pathways activated in this step of the parasite life cycle. However, it is notable that

TABLE 4 Inhibition of *P. falciparum* oocyst formation by pyronaridine added to gametocytes 24 or 48 h before *A. stephensi* feeding[a]

Time (h)	Pyronaridine concn (μM)	No. of mosquitoes infected/total no. of mosquitoes	Estimated % inhibition of oocyst counts (95% CI [low, high])	*P* value
48	0.4	15/20	66.7 (27.0, 85.6)	0.005
	0.05	19/20	10.7 (−112.1, 63.5)	0.821
24	0.4	15/20	50.5 (−14.0, 78.2)	0.097
	0.05	20/20	−37.2 (−213.6, 37.7)	0.459

[a]Data are from *P. falciparum* SMFAs with compounds added to parasites 24 or 48 h before mosquito feeding.

compounds targeting cell replication pathways are prominent, likely due to the need for three mitotic events for the formation of male microgametes (2). Of the 185 validated compounds, 7 were chosen for further evaluation based on their activity against asexual stages, putative modes of action, and history of use as antimalarials. Of these, only two of the seven, batimastat and quinacrine, had no significant activity against mosquito infections with either *P. falciparum* or *P. vivax*, highlighting the predictive power of *P. berghei* Ookluc in identifying TB compounds. Interestingly, the most active compounds against mosquito infection also have strong activity against exflagellation, confirming previous conclusions that the early steps in *Plasmodium* gametogenesis are potentially effective drug targets (22) and suggesting that the development of a high-throughput screening tool with an exflagellation reporter may be an attractive future approach.

Efforts to find new medicines for malaria control can be guided by target candidate profiles (TCPs), dividing types of compounds according to their activities against the parasite (22). One important TCP aims to target the sexual stages, with a goal of 90% transmission-blocking activity in mosquitoes 7 days after oral dosage, which is the mean circulation time of mature gametocytes and half the duration of gametocyte carriage after treatment with artemisinins (22). Therefore, new TB compounds with an intrinsically long half-life or chemically developed for a long half-life may be proposed as a component of future artemisinin-based combination therapy (ACT) to prevent parasite transmission to mosquitoes after the clearance of asexual stages. Moreover, they may be used in the development of new antimalarials for mass drug administration for reducing malaria transmission in specific epidemiological settings. Another potential use is in baits or nets to render the mosquitoes refractory to infection, as demonstrated previously for atovaquone (23).

Hesperadin was the most active compound against mosquito infections, with strong inhibition of *P. falciparum* oocyst formation at nanomolar concentrations. Hesperadin is a putative inhibitor of human Aurora kinases. In human cells, Aurora kinases are important in cellular division, controlling chromatid segregation (24). *Plasmodium* parasites express three Aurora kinases (25), with only a modest identity (~40%) to the human genes in the kinase domains. Hesperadin blocks nuclear division in *P. falciparum* (26), an effect that supports Aurora kinases as the putative target in the parasite. Among the 185 validated potential TB compounds, at least 3 other putative Aurora kinase inhibitors were identified in addition to hesperadin. These results support the further evaluation of Aurora kinases as potential TB antimalarial drug targets.

DDD107498 is a *P. falciparum* elongation factor 2 inhibitor with activity against multiple stages of the parasite (27). It is currently in clinical development with the name M5717. The TB activity of DDD107498 against *P. falciparum* confirmed previous results (27, 28), and our results for *P. vivax* infections of *Anopheles darlingi* mosquitoes showed comparable TB activities, highlighting the potential use of DDD107498 in reducing malaria transmission.

Pyronaridine, a benzonaphthyridine derivative, has potent activity against *Plasmodium* asexual stages, targeting hematin formation in the parasite (29). Pyronaridine has a long elimination half-life, up to 13 days in malaria patients (30), and is an approved antimalarial given in combination with artesunate under the brand name Pyramax (180 mg pyronaridine and 60 mg artesunate). Previous studies assessed the TB potential of pyronaridine and found low activity in gametocyte viability or gametogenesis assays (31–33). At least one study evaluated pyronaridine in *P. falciparum* SMFAs, preincubating the gametocytes with 1 μM the drug for 24 h, and found 80% oocyst reduction (34), comparable to the results that we observed by adding the drug immediately prior to mosquito feeding. We also demonstrate that pyronaridine is not active against *P. berghei* male gametogenesis, in line with previous findings that it is poorly active against mature stage V *P. falciparum* gametocytes (34), and we show that it likely inhibits parasite transmission to mosquitoes by targeting the zygotes formed in the mosquito midgut. Whether the inhibitory mechanism in zygotes is also through targeting heme metabolism remains uncertain, but the fact that pyronaridine was inactive against *P. vivax* transmission suggests that the target of

P. falciparum sexual stages may be different from the inhibitory mechanism described for asexual stages since in this case, the drug is active against both parasite species. In pyronaridine-artesunate-treated patients, pyronaridine has a long half-life, with blood concentrations ranging from ~400 nM in the first 24 h after administration to ~50 nM after 7 days (16, 35, 36). Thus, we tested these concentrations in *P. falciparum* SMFAs by incubating the drug with the gametocytes before mosquito feeding. Our results for oocyst inhibition with 400 nM pyronaridine after 48 h of incubation suggest the possibility of an impact on transmission in places where pyronaridine-artesunate was already implemented. These results also support the future design of clinical studies evaluating whether pyronaridine administered at doses of >180 mg can provide blood concentrations that will have an important impact on transmission.

In conclusion, the *in vitro* screens using the *P. berghei* Ookluc line were able to identify compounds that are active against *P. falciparum* and *P. vivax* sexual stages, revealing activity against the first stages of these parasites in the mosquito midgut. In some cases, this activity adds to previously known activities against asexual stages and gametocytes. In other cases, the Ookluc screen revealed the antimalarial activity of compounds that are not active against asexual stages. Our list of compounds with transmission-blocking potential can thus serve as a guide for future studies for developing antimalarials that are active against multiple parasite stages.

MATERIALS AND METHODS

Animals and parasite strains. C57BL/6 or BALB/c mice were bred and maintained in the animal facility of the Department of Parasitology at the Institute of Biomedical Sciences, University of São Paulo, under protocol 921503119-CEUA of research ethics approval for animal experimentation or in the NIH animal facility under protocol LMVR 10E. The *P. berghei* Ookluc line was stored as frozen stocks in liquid nitrogen or at −80°C. Vial stocks were prepared by mixing 150 μL of parasitized mouse blood with 300 μL of Alsever's solution (catalog number A3551; Sigma-Aldrich) with 10% glycerol (catalog number G5516; Sigma-Aldrich). Mice were infected by the intraperitoneal injection of 200 μL of thawed stocks. Parasitemia was monitored daily by Giemsa staining (Laborclin [catalog number 620529] or MilliporeSigma [catalog number GS500]) of thin blood smears on glass slides (catalog number K5-7105-1; Kasvi) with counting by direct light microscopy with a 100× oil immersion objective (Nikon E200).

Conversion assays. For conversion assays, in which gametocytes convert into gametes and fertilize to form zygotes *in vitro*, parasitized mouse blood was obtained by cardiac puncture in heparinized syringes (heparin sodium salt, catalog number H3393; Sigma-Aldrich) and added to ookinete medium (1:10 dilution). The ookinete medium (37) consisted of RPMI 1640 medium (catalog number 61870; Thermo Scientific) with 0.025 M HEPES (catalog number 15630080; Thermo Scientific), penicillin-streptomycin-neomycin (catalog number P4083; Sigma-Aldrich), 50 mg/L hypoxanthine (catalog number H9636; Sigma-Aldrich), and 100 μM xanthurenic acid (catalog number D120804; Sigma-Aldrich) (pH 8.3).

For assays in 1,536-well plates, the plates were prepared with compound dilutions in 4 μL of ookinete medium in each well. Parasitized blood was first diluted 1:1 in phosphate-buffered saline (PBS) at 37°C, and 1 μL of the resulting solution was then dispensed into each well with the aid of an automated dispenser (MultiDrop Combi reagent dispenser; Thermo Fisher Scientific). The plates were kept at 21°C in an incubator for 6 h, and the luciferase activity was determined by measuring relative light units (RLU) using a ViewLux μHTS microplate imager (Perkin Elmer) after the addition of 1 volume of the substrate/lysis buffer (Nano-Glo luciferase assay system; Promega). For phenotypic assays, 2 μL of parasitized blood was added to 20 μL of ookinete medium in 0.6-mL tubes, and the assay mixtures were kept at 21°C in an incubator for 1 and 6 h. The luciferase activity was determined by measuring the RLU using a microplate reader (POLARstar Omega; BMG LabTech) after the addition of 1 volume of the substrate/lysis buffer (Nano-Glo luciferase assay system; Promega). For some experiments, lysis buffer was not added so that the zygotes were not destroyed and could be counted by blood smears made with 2 μL of the blood cells at the bottom of the tube. Primary screening hits were confirmed with three independent replicates of freshly prepared, serially diluted compounds. For exflagellation assays, 4 μL of parasitized blood was added to 16 μL of ookinete medium containing the test compound, the mixture was incubated at 21°C for 15 min, and the exflagellation centers were counted in 20 40× fields under a light microscope.

***Plasmodium* asexual-stage assays.** The *P. falciparum* parasite Dd2 line was cultured *in vitro* under standard conditions (38). Briefly, parasites were maintained in 2% human O^+ erythrocytes (Interstate Blood Bank, Memphis, TN) in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 0.5% Albumax II (Life Technologies), 2 mM L-glutamine, 50 mg/L hypoxanthine, 25 mM HEPES, 0.225% $NaHCO_3$, 24 mM sodium bicarbonate, and 10 μg/mL gentamicin. Tissue culture flasks and assay plates were incubated at 37°C under a gas mixture of 5% CO_2, 5% O_2, and 90% N_2. Methods for SYBR green I qHTS (quantitative high-throughput screening), the calculation of the AC_{50} (activity concentration 50%), and the definition of curve classes were described previously (39–41). qHTS assays were performed using an 11-point concentration-response format, with 72 h of drug exposure. Percent response values represent relative growth as judged by SYBR green I fluorescence intensity values normalized to the values of the controls.

Assay counterscreens. To assess toxicity against mammalian cells, 2×10^3 HepG2 cells per well were dispensed into white solid-bottom 1,536-well plates in Dulbecco's modified Eagle's medium (DMEM) using a MultiDrop Combi dispenser in a 5-μL volume, as described previously (42). Compounds dissolved in dimethyl sulfoxide (DMSO) were transferred to the assay plate via acoustic droplet ejection (Echo 655; Labcyte, San Jose, CA), with a final concentration range of 1.6 nM to 57 μM. Each plate included medium-only (no cells) and solvent-only control wells in columns 1 to 4. The plates were incubated for 48 h at 37°C, and 1 volume of CellTiter-Glo assay reagent (Promega, Madison, WI) was added using a BioRAPTR flying reagent dispenser (FRD) (Beckman Coulter, Brea, CA). Cell viability was measured using a ViewLux μHTS microplate imager.

To evaluate potential nanoluciferase reporter interference compounds, medium from nLuc-expressing S16 cells was filtered through a 0.22-μm filter (43). Two microliters of the nLuc assay substrate (Nano-Glo luminescence assay; Promega, Madison, WI) was dispensed into each well with a BioRAPTR FRD, and compounds were acoustically transferred to the plate with an Echo 655 instrument in concentration-response 11-point dilutions. After 5 min of incubation of the substrate and compound at room temperature, 2 μL of filtered nLuc-containing medium was added to each well with a BioRAPTR FRD, the mixture was incubated for 20 min, and luminescence was assessed using a ViewLux μHTS microplate imager.

***P. falciparum* standard membrane-feeding assay.** The standardized methodology for performing the SMFA was described previously (44). Briefly, 16- to 18-day-old gametocyte cultures of the *P. falciparum* NF54 line (200 μL of a 50% hematocrit culture adjusted to 0.15 to 0.2% stage V gametocytemia) were mixed with 60 μL of a test sample, and the final mixture was immediately fed to ~50 female *Anopheles stephensi* (Nijmegen strain, 3 to 6 days old) mosquitoes through a membrane-feeding apparatus. Mosquitoes were kept for 8 days and dissected (n = 20 per group) to enumerate the oocysts in the midgut. Only midguts from mosquitoes with any eggs at the time of dissection were analyzed (60 to 80% of mosquitoes were egg positive in general). The human serum and red blood cells used for the gametocyte cultures and feeding experiments were purchased from the Interstate Blood Bank.

***P. vivax* direct membrane-feeding assay.** Patients visiting malaria clinics in Porto Velho, Rondônia, northwestern Brazil, were microscopically examined for malaria infections. When patients were diagnosed with *P. vivax* infection, if they were ≥18 years old and not pregnant, they were invited to donate blood for *P. vivax* DMFAs. The protocol for blood collection was approved by the Ethical Committee of the Centro de Pesquisa em Medicina Tropical (CEPEM), Rondônia, Brazil (protocol number 28176720.9.0000.0011), and written, informed consent was obtained from all volunteers. The blood samples were kept at 37°C using a temperature-controlled container during blood transportation and a water bath during blood processing and mosquito feeding. Less than 30 min after collection, heparinized blood was aliquoted at 2 mL per tube before being centrifuged, the plasma was removed, and a pool of normal human AB^+ serum (50% hematocrit) was immediately placed into feeding apparatuses and offered to *A. darlingi* mosquitoes from a Brazilian colony (45). Two milliliters was offered to 60 female mosquitoes. Control groups were fed with infected blood containing the DMSO vehicle. Experimental groups were fed with infected blood containing 10, 2, or 0.4 μM the compound. The mosquitoes were allowed to feed on infected blood for 30 min. After the removal of unfed mosquitoes, the remaining mosquitoes were kept at the insectary at 26°C ± 1°C with a relative humidity of 70% ± 10% and fed daily on a 15% honey solution. At 7 days postinfection, the mosquitoes were dissected, and their midguts were stained with 0.2% commercial mercurochrome and examined for the presence of oocysts to determine the prevalence and infection intensity.

Statistical and comparative analyses. Student's t tests were used to calculate statistical differences between sample groups. The Z-factor for the high-throughput assay was calculated as described previously (46), with positive controls being wells for conversion assays as described above and negative controls being wells for conversion assays with nonparasitized blood or wells with nonactivated Ookluc (in both cases, the RLU signal is the same).

For SMFAs and DMFAs, the best estimate of the percent inhibition of the oocyst density percent of transmission-reducing activity (%TRA) from multiple feeds, the 95% confidence interval of the %TRA, and the P value were calculated using a zero-inflated negative binomial model (44).

Data availability. All data are available in the text or the supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 1.3 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.02 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.03 MB.

SUPPLEMENTAL FILE 4, PDF file, 1.4 MB.

SUPPLEMENTAL FILE 5, XLSX file, 1.3 MB.

SUPPLEMENTAL FILE 6, XLSX file, 0.02 MB.

SUPPLEMENTAL FILE 7, XLSX file, 0.03 MB.

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We declare no competing interests.

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