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SUSCEPTIBILITY

Screening the Pathogen Box for Molecules Active against *Plasmodium* Sexual Stages Using a New Nanoluciferase-Based Transgenic Line of *P. berghei* Identifies Transmission-Blocking Compounds

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ABSTRACT Malaria remains an important parasitic disease with a large morbidity and mortality burden. *Plasmodium* transmission-blocking (TB) compounds are essential for achieving malaria elimination efforts. Recent efforts to develop high-throughput screening (HTS) methods to identify compounds that inhibit or kill gametocytes, the *Plasmodium* sexual stage infectious to mosquitoes, have yielded insight into new TB compounds. However, the activities of these compounds against gametes, formed in the first minutes of mosquito infection, are typically not assessed, unless screened in a standard membrane feeding assay, a labor-intensive assay. We demonstrate here the generation of a *Plasmodium* model for drug screens against gametes and fertilization. The new *P. berghei* line, named *Ookluc*, was genetically and pharmacologically validated and scalable for HTS. Screening the Pathogen Box from the Medicines for Malaria Venture using the new model identified promising TB compounds. The use of *Ookluc* in different libraries of compounds may aid in the identification of transmission-blocking drugs not assessed in screens against asexual stages or gametocytes.

KEYWORDS HTS, Pathogen Box, antimalarial agents, gametes, malaria, transmission

In 2016, nearly half of the world population was at risk of malaria, and 216 million cases of the disease killed 445 thousand people (1). Malaria is transmitted to humans through the bite of infected *Anopheles* mosquitoes, and the symptomatic disease is caused by the multiplication of asexual stages of species of *Plasmodium* within erythrocytes. The transmission cycle is completed when mosquitoes ingest the sexual gametocyte stage upon biting an infected individual. When ingested during a mosquito blood meal, they activate and transform into male and female gametes in the mosquito midgut. Within the first hour, the gametes fertilize to form a zygote, which after 18 to 24 h becomes an ookinete (a motile zygote) (2). The ookinete invades the midgut epithelium and then settles at the basal lamina to form an oocyst (2). In the oocyst, thousands of sporozoites develop and bud after 12 to 16 days to migrate to the mosquito salivary glands (3), where they wait to be injected to the vertebrate host with another blood meal.

Malaria control is largely based on vector control with the use of insecticidal nets and long-lasting indoor residual insecticide spraying to protect humans from mosquito bites. Recent reports of mosquito resistance to insecticides are a matter of concern (4). The use of artemisinin combination therapies can efficiently clear the circulating asexual-stage parasites, with some antimalarials also possessing activity against the

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transmissible gametocyte. However, the emergence and spread of drug-resistant parasites in Asia reduce the effectiveness of the combination therapy and reduce their use for mass drug administration to reduce transmission (5).

The problem of mosquito and parasite resistance has led the World Health Organization (WHO) to launch, in 2015, *The WHO Global Technical Strategy for Malaria 2016-2030* (http://www.who.int/malaria/en/), which aims at reducing the malaria incidence by 90% by 2030 and to eliminate malaria (interrupt local transmission) in at least 35 countries. One of the specific aims of the global strategy is to implement transmission-blocking (TB) chemotherapy.

The only drug approved for TB chemotherapy is primaquine. However, its use is limited by toxicity concerns, especially in patients carrying deficient alleles of the glucose-6-phosphate dehydrogenase (G6PD) enzyme. Thus, developing new TB antimalarials is important for malaria control and elimination efforts.

Efforts to identify and develop new TB compounds utilize high-throughput screening (HTS) methodologies that assess gametocytocidal activity (6). Continuous cultures of some lines of *Plasmodium falciparum* are compatible with gametocyte formation. Systems accessing gametocytogenesis or gametocyte clearance (7–14) and gametocyte viability (15, 16) in high throughput (HT) or semi-HT are available. However, to date only two models, developed using *Plasmodium berghei*, are able to assess gametocyte function, i.e., gametogenesis, zygote formation, and ookinete morphogenesis, in semi-HT (17) or HT (18).

Here, we present the generation of a *P. berghei* model to assess gametocyte function in HT and the application of the new model to identify TB compounds in the Pathogen Box from the Medicines for Malaria Venture (MMV).

RESULTS

Generation of *Ookluc*. To generate a new *Plasmodium* experimental model for HT assessment of gametocyte function, we used the *P. berghei* line Gene In/Marker Out (GIMO) (19) to insert an expression cassette for nanoluciferase (nLuc; Promega) expression under the control of the ookinete-specific promoter, *P. berghei* CTRP (20) (Fig. 1a). Transfected parasites were negative selected with 5-fluorocytosine (5-FC), and the resulting mutants, named *Ookluc*, retain no selectable markers in the genome.

Circulating gametocytes in *P. berghei*-infected mouse blood can activate, form gametes, fertilize to form a zygote, and transform into ookinetes *in vitro* in conversion assays using ookinete medium (21). The assay to verify *Ookluc* conversion and nLuc activity combines infected mouse blood dispensed in ookinete medium at 21°C. After 0, 6, or 24 h, the nLuc substrate (Nano-Glo) was added with lysis buffer at 1:1 (vol/vol) to measure the emitted luminescence (relative light units [RLU]) in a plate luminometer (Fig. 1b). *Ookluc* had negligible nLuc activity in circulating blood stages (time zero, no incubation), and in *in vitro* fertilization assays (conversion) the signal has a relative increase of ~40-fold after 6 h and ~2,000-fold after 24 h (Fig. 1c), demonstrating that the *ctrp* promoter is sufficiently silenced to prevent detectable nLuc expression in blood stages and is activated only upon conversion into mosquito sexual stages. The nLuc activity after 24 h conversion directly correlated with the gametocytemia of the mouse used as blood donor for the assay (see Fig. S1 in the supplemental material) and with the number of formed ookinetes counted by blood smears (Fig. 1d), and the best assays regarding signal/background ratio, which was around 2,000, were achieved utilizing blood with gametocytemia above 0.4%.

Validation of *Ookluc*. To verify whether the increase in nLuc activity in conversion assays with *Ookluc* was dependent on fertilization, we deleted the *mtrap* gene from the *Ookluc* genome. MTRAP-knockout (MTRAP^{KO}) gametocytes can activate, but the gametes remain trapped inside the host cell (22). There was no increase in nLuc activity in *Ookluc*-MTRAP^{KO} parasites after 24 h of conversion assay (Fig. 2a)—the absence of ookinetes was confirmed by blood smears (not shown)—showing that gametocyte activation *per se* is not sufficient for activation of the *ctrp* promoter; hence, nLuc expression and activity in *Ookluc* parasites depend on gamete egress and fertilization.

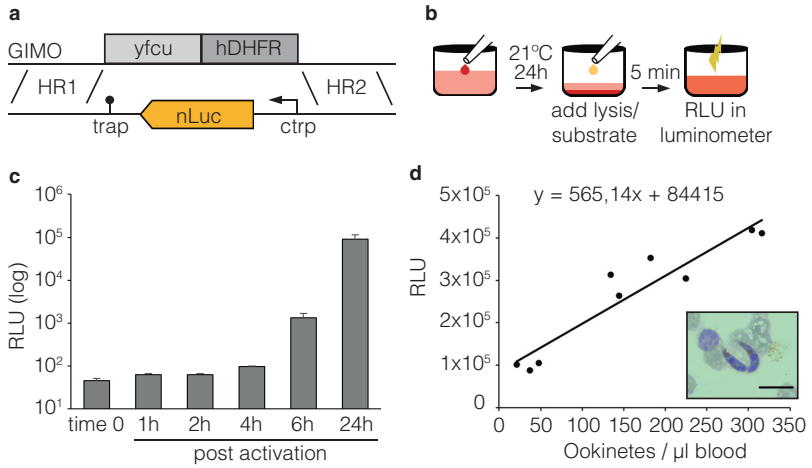

FIG 1 Generation of the *Ookluc* line. (a) Strategy for generating the *Ookluc* reporter line. The coding sequence of nLuc (orange box) was cloned downstream the *ctrp* promoter (arrow) and upstream the 3′ UTR of the thrombospondin-related adhesive protein (TRAP; lollipop) into the plasmid pL0043 (19), containing the homology regions (HRs) for integration in the GIMO locus. Gray boxes indicate the resistant markers yeast fcu (yfcu) and human dihydrofolate reductase (hDHFR). (b) Ookinete conversion assay. Blood from an infected mouse is dispensed in a well of 96-well plate containing ookinete medium at 21°C and incubated for 24 h. Then, nLuc substrate is added, and the relative light units (RLU) were assessed by using a plate luminometer after a 5-min incubation. (c) Kinetics of nLuc activity, expressed as RLU (means + the SD), in *Ookluc* blood stages (blood) or different time points postactivation in conversion assay. The results are representative of 5 independent experiments. (d) Correlation between the number of ookinetes formed per μl of blood after 24 h conversion and the measured RLU. The RLU was measured without addition of lysis buffer, and the ookinetes were then counted by blood smears (for the image displayed: scale bar, 10 μm). Linear trendline and equation are shown. The results are representative of three independent experiments.

Likewise, blocking gametogenesis with the phospholipase C inhibitor U73122 (23) reduced nLuc activity in *Ookluc* after 24 h of conversion assay; in contrast, the inactive analog U73343 or dimethyl sulfoxide (DMSO) had no effect (Fig. 2b). A reduction of ookinete formation with U73122 was confirmed by blood smears (data not shown). These results validate *Ookluc* as a tool for searching compounds with TB activity through inhibition of gametocyte activation, gametogenesis, or fertilization.

The increase in nLuc activity after 6 h of conversion assay with *Ookluc* showed that the *ctrp* promoter likely becomes active soon after zygote formation. Inhibition of palmitoylation with 2-bromopalmitic acid (2-BP) 1 h after *Ookluc* activation, blocking zygote to ookinete morphogenesis (24), did not reduce the nLuc activity after 6 h (Fig. 2c) or 24 h (Fig. 2d). This indicates that once the *ctrp* promoter is activated in zygotes it accumulates nLuc regardless of ookinete formation and precludes *Ookluc* as a tool for searching compounds with TB activity through inhibition of ookinete morphogenesis. The activity of 2-BP was validated by its effect on *Ookluc* conversion when added on time zero of the assay, completely blocking the nLuc signal after 6 h (Fig. 2c) or 24 h (Fig. 2d)—the absence of ookinetes was confirmed by blood smears (data not shown)—and revealing an essential role of palmitoylation in the first steps of *Plasmodium* sexual development in mosquitoes, i.e., gametocyte activation, gametogenesis, or fertilization.

The *Ookluc* conversion assay is scalable for HTS. The level of variation of nLuc activity in conversion assays with *Ookluc* in a 96-well plate format represented a normal distribution, with 93.7% of the reads within the means ± two standard deviations (SD; Fig. S2a), and with a Z-factor of 0.6 (25), validating *Ookluc* as a tool for HTS. Different volumes of infected blood and medium can be used for the conversion assays, yielding robust RLU signals after 24 h (Fig. S2b). Importantly, assays with a reduced total volume (5 to 11 μl) are compatible with volume limitations of both 384- and 1536-well format assays, the prominent format used in most HTS compound libraries, while also reducing animal use and reagent expense.

Screening the Pathogen Box with *Ookluc* identifies TB compounds. We then used the *Ookluc* model to screen for compounds with TB activity in the Pathogen Box

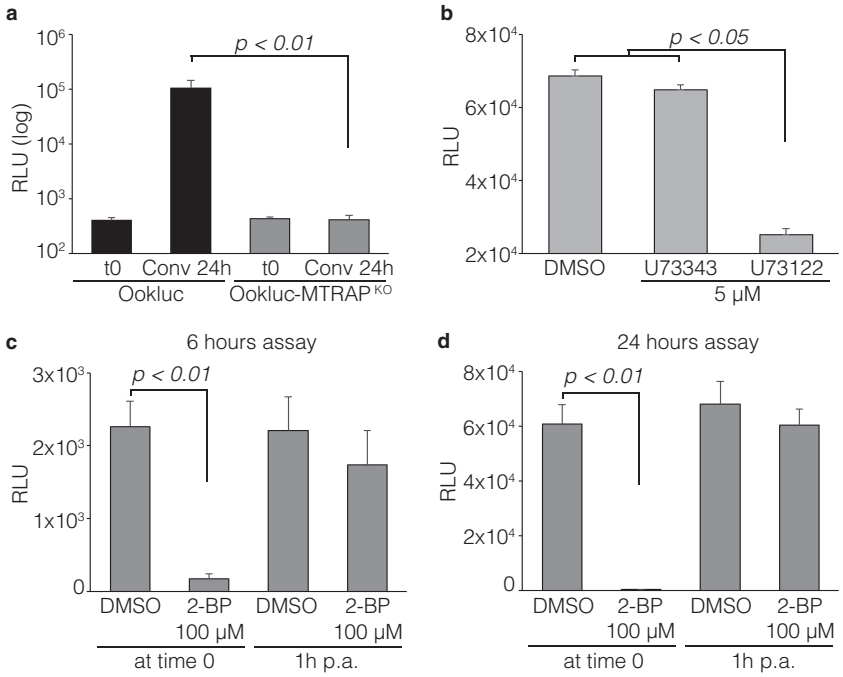

FIG 2 nLuc activity in conversion assay with *Ookluc* depends on fertilization. (a) nLuc activity, expressed as RLU, in *Ookluc* and *Ookluc*-MTRAP^{KO} blood stages (t_0) or after 24 h of conversion assay. (b) nLuc activity, expressed as RLU, in *Ookluc* parasites after 24 h of conversion assay in the presence of 5 μM the phospholipase C inhibitor U73122 or its inactive analog U73343. Control, DMSO. (c and d) nLuc activity, expressed as RLU, in *Ookluc* parasites after 6 h (c) or 24 h (d) of conversion assay in the presence of 100 μM 2-BP, an inhibitor of palmitoylation, or DMSO control, added at time zero or 1 h postactivation (p.a.). All bars show the means + the SD and are representative of four independent experiments.

(Medicines for Malaria Venture [MMV]). Compounds were initially tested at a concentration of 10 μM. From the ~400 compounds in the Pathogen Box, 31 showed more than 95% inhibition of conversion, 45 showed more than 90% inhibition, and 75 showed more than 75% inhibition (Dataset S1). This screen identified compounds with TB potential and importantly demonstrated some overlap and divergence from a previous screen (26) against late-stage *P. falciparum* gametocytes (LSG; data kindly provided by V. Avery) (Fig. 3) and previous screens for compounds that existed in the MMV Malaria Box (https://chembl.gitbook.io/chembl-ntd/) (Fig. S3). This suggests the conservation of some drug targets between gametocytes and gametes, as well as the emergence/loss of additional targets between these closely related stages (Fig. 3). Moreover, the integration of the *Ookluc* and *P. falciparum* LSG inhibition data with the data available from MMV regarding inhibition of *P. falciparum* asexual growth and *P. berghei* liver stages provides a comprehensive view of compound potential against *Plasmodium* stages (Fig. S3), an important tool to guide drug development by prioritizing compound classes. The 31 compounds inhibiting more than 95% of conversion at 10 μM were then tested at a final concentration of 1 μM, and those showing more than 95% inhibition at this lower concentration were subsequently screened using an initial concentration of 1 μM and serial 2-fold dilutions to determine the 50% inhibitory concentration (IC_{50}) (Fig. 4). The nine most potent compounds (Fig. 4) did not inhibit nLuc activity in *P. falciparum* blood stages expressing nLuc (27) at a final concentration of 10 μM (Fig. S4a), and inhibition of ookinete formation was confirmed by direct blood smears of *P. berghei* conversion assays (Fig. S4b), confirming that their inhibitory activity in *Ookluc* conversion assays is not due to direct inhibition of nLuc.

DISCUSSION

This study presents a new *P. berghei* model, *Ookluc*, to study *Plasmodium* transmission. At least one parasite line similar to *Ookluc* has been previously reported (18). It

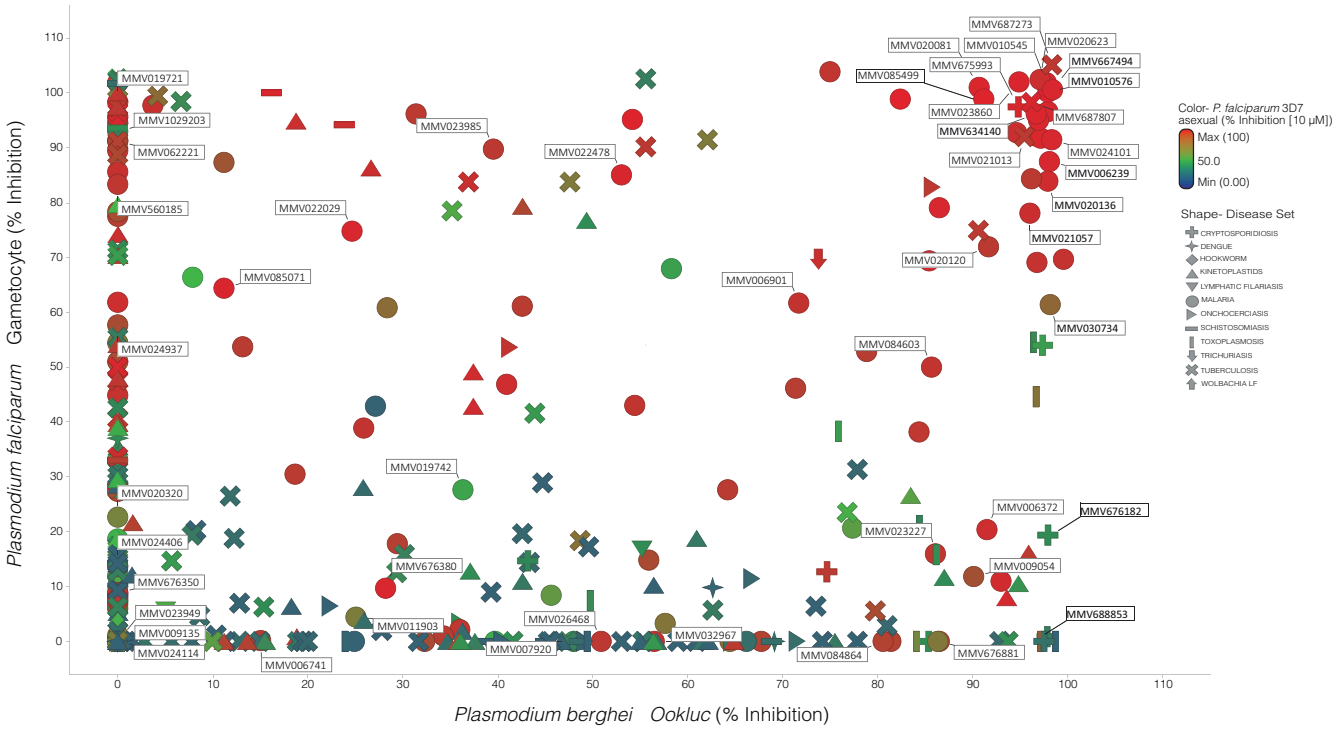

FIG 3 Multidimensional scatterplot of *P. berghei Ookluc* activity compared to *P. falciparum* gametocyte (stage IV and V) inhibition. The comparative activity of the MMV Pathogen Box compounds, previously screened as part of the MMV Malaria Box, is shown. The data represent the percent inhibition of the compounds utilizing the *Ookluc* assay screened at a single 10 μM concentration compared to the percent inhibitory activity, again at a single 10 μM concentration, against *P. falciparum* late-stage gametocytes (stages IV and V) (26). Colors reflect the growth inhibition level against *P. falciparum* 3D7 asexual parasites screened at 10 μM compound concentration, from blue (0% growth inhibition) to red (100% growth inhibition) (grey indicates no value). The symbols indicate the different annotated disease sets within the Pathogen Box.

uses a fluorescence reporter under the control of *ctrp* promoter, and the selectable marker for insertion of the reporter remains in the parasite genome. *Ookluc* has important improvements; specifically, the use of nLuc, a more sensitive reporter, and the absence of an integrated drug resistance marker make *Ookluc* ideal for drug screenings.

While most methodologies for screening TB compounds focus on gametocyte cultures and thus identify gametocytogenesis inhibitors or gametocytocidal compounds, the *Ookluc* model can assess parasites viability through ability to progress along the life cycle and complete sexual recombination. Blocking palmitoylation at 1 h after conversion culture (after gametogenesis and fertilization have occurred) did not impact the nLuc signal, showing that inhibitory compounds identified using the *Ookluc* model are likely acting prior to zygote formation. Therefore, *Ookluc* can identify TB compounds with activity against gametogenesis or sexual recombination, as well as those potentially killing mosquito stages, like reactive oxygen species inducers.

Unlike multiplicative *Plasmodium* asexual stages, late-stage gametocytes (LSG) are metabolically quiescent. Active metabolic pathways differ between these stages. Accordingly, >90% of antimalarial compounds active against asexual stages have no activity against LSG (28). Likewise, the transition from gametocytes to gametes and zygotes involve profound changes in metabolic activity (29), predicting that inactive compounds against gametocytes may be active against gametes and zygotes, or vice versa. Indeed, assays using *Ookluc* identified TB compounds previously not assessed in other screens against LSG. Therefore, integrating data obtained using the *Ookluc* model with data from screens against asexual stages, LSG and liver stages can provide valuable information for prioritizing compound classes active against all parasite stages. Moreover, *Ookluc* can be used as a tool for chemical genomics efforts in order to probe metabolic pathways that differ in parasite stages progressing through the life cycle.

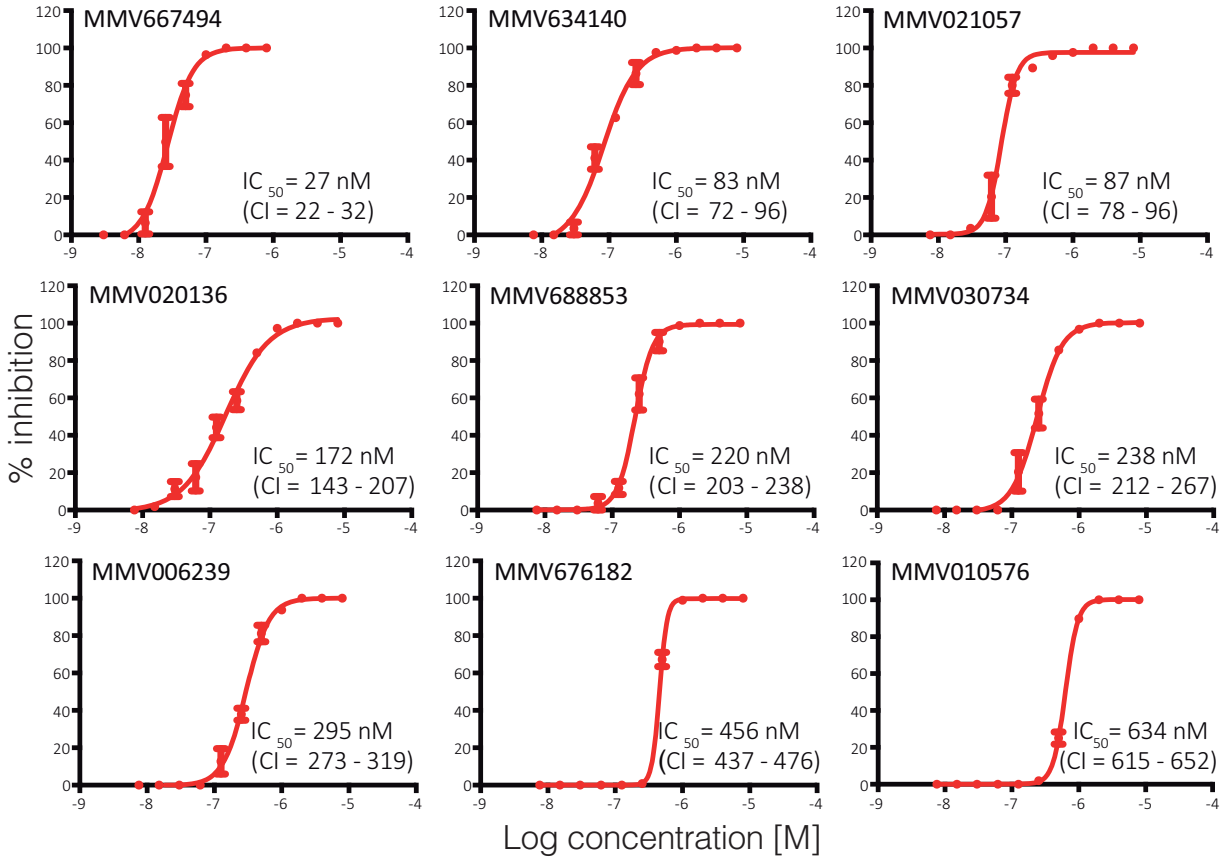

FIG 4 Dose-response curves and determination of IC_{50}. Indicated compounds were 2-fold serially diluted in conversion assays, in triplicates, starting from 1 μM (100% inhibition) until no inhibition was observed. The calculated IC_{50} and top and bottom 95% confidence intervals (CI) for each compound are indicated in the graphs. Inhibition at each dilution point is shown as means $\pm$ the SD. The results are representative of three independent experiments.

Screening the Pathogen Box from MMV with *Ookluc* identified nine potent TB compounds with IC_{50} at the nM range. The compound MMV010576, with structure similarity to previously described kinase inhibitors (CHEMBL5903 and CHEMBL5311; Tanimoto value of 0.79), has also reported potent activity against asexual stages, LSG and liver stages, and thus represents a promising starting point for the development of compounds targeting at least four parasite developmental stages. The screen identified other known kinase inhibitors. MMV030734 is a specific inhibitor of *Plasmodium* calcium-dependent protein kinase 1 (CDPK1) (30), which is critical for parasite gametogenesis (31). MMV676182 and MMV688853 are bumped kinase inhibitors active against *Toxoplasma* and *Cryptosporidium* CDPK1 (32). The ortholog of TgCDPK1 in *Plasmodium* is CDPK4, a regulator of gametogenesis essential for parasite transmission (33). Analogs of MMV676182 were shown to specifically inhibit PfCDPK4 and block malaria transmission (34). The quinoline 4-carboxamides MMV667494 and MMV634140, inhibitors of *Plasmodium* translation elongation factor 2 (35), represent another class of compounds also identified as potent TB using *Ookluc*. They are related to the compound DDD107498, a potent multistage antimalarial with known TB activity (36). These potent TB compounds identified using *Ookluc* were previously identified using different methodologies. The ability of *Ookluc* screens to identify these compounds validate the model as a powerful tool for further screens using available libraries and identification of novel TB antimalarials.

Thus, we present here a powerful new tool to run HTS against *Plasmodium* sexual stages and find new TB compounds that may aid in the ambitious goal of eliminating malaria in the next 15 years.

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 Sao Paulo, under the protocol 132/2014-CEUA of research ethics approval for animal experimentation. The *P. berghei* ANKA recombinant Gene Insertion/Marker Out (GIMO) line (19) and the new *Ookluc* line were 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 (Sigma-Aldrich, A3551) with 10% glycerol (Sigma-Aldrich, G5516). Mice were infected by intraperitoneal injection of 200-μl portions of thawed stocks. The parasitemia was followed daily through Giemsa staining (Laborclin, 620529) of thin blood smears in glass slides (Kasvi, K5-7105-1) counted by direct light microscopy with a 100× oil immersion objective (Nikon E200).

Construction of plasmids. To construct the plasmid for generating the *Ookluc* strain, the 1,590 bp upstream the genomic sequence of the *P. berghei* "circumsporozoite protein and thrombospondin-related adhesive protein [TRAP]-related protein" (CTRP, PBANKA_0412900) were PCR amplified using forward (GGGCTGCAGCCACTTCCTCAAAATGAATAGG (the PstI restriction site is underlined) and reverse (GGATCCTTGTGTTTTGCTTTGTATTTAAA; the BamHI restriction site is underlined) primers. The coding sequence of nanoluciferase (nLuc) was PCR amplified from the plasmid PfNluc (27) using forward (GGATCCATGGTCTTCACACTCGAAG; the BamHI restriction site is underlined) and reverse (GATATCT-TACGCCAGAATGCGTTCG; the EcoRV restriction site is underlined) primers. The 551-bp downstream 3′ untranslated region (3′ UTR) genomic sequences of the *P. berghei* thrombospondin-related adhesive protein (TRAP, PBANKA_1349800) were PCR amplified using forward (GATATCTTTTAATAAACATATATAT CTAGAT; the EcoRV restriction site is underlined) and reverse (GCGGCCGCCATCGCTGCATTAATGATTT; the NotI restriction site is underlined) primers. The amplified sequences were cloned into plasmid pL0043 (19), which is designed for transfection of the *P. berghei* GIMO line, using the PstI, BamHI, EcoRV, and NotI restriction sites (FastDigest; Thermo Scientific) to generate plasmid p43-Ookluc. The plasmid for knocking out the merozoite thrombospondin-related adhesive protein (MTRAP; PBANKA_0512800) was already available (22).

Parasite transfection. For the transfections, the plasmids were electroporated into synchronized *P. berghei* asexual schizonts using an Amaxa (Lonza) Nucleofector electroporator set at program U33 and using a human T cell Nucleofector kit (Lonza, VPA-1002) as previously described (37).

For transfection of the *P. berghei* GIMO, plasmid p43-Ookluc was linearized with SacII (FastDigest; Thermo Scientific), and transformed parasites were negative selected by the intraperitoneal administration of one dose per day of 0.4 g/kg (body weight) of 5-fluorocytosine (Sigma-Aldrich, F7129) to mice infected with the transfected parasites, starting 24 h after transfection.

To knock out *mtrap* from *Ookluc*, the sequence targeting the *mtrap* locus for replacement of the coding sequence by cassettes for mCherry fluorescence and pyrimethamine resistance was removed from a plasmid previously available (22). After transfection, transformed parasites (*Ookluc*-MTRAP^{KO}) were positive selected by the administration of pyrimethamine (Sigma-Aldrich, 46706) for 72 h in the drinking water (70 mg/liter) to mice infected with the transfected parasites, starting 24 h after transfection. After selection, the transformed parasites were cloned by limiting dilution and infection of mice.

Conversion assays. For conversion assays, parasitized mouse blood was obtained by cardiac puncture in heparinized syringes (heparin sodium salt from Sigma-Aldrich, H3393) and added to ookinete medium (1/20). The ookinete medium (21) consisted of RPMI 1640 (Thermo Scientific, 61870) with 0.025 M HEPES (Thermo Scientific, 15630080), penicillin-streptomycin-neomycin (Sigma-Aldrich, P4083), hypoxanthine 50 mg/liter (Sigma-Aldrich, H9636), and xanthurenic acid 100 μM (Sigma-Aldrich, D120804) at a pH of 8.3.

The assays were kept at 21°C in an incubator for 0, 6, or 24 h. The luciferase activity was determined by measuring the RLU using a microplate reader (SpectraMax i3; Molecular Devices) 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 the cells are not destroyed and can be counted by blood smears. The phospholipase C inhibitor U73122 (Sigma-Aldrich, U6756) and the inactive analog U73343 (Sigma-Aldrich, U6881) were used at final concentrations of 5 μM, which blocks gametocyte activation (23). The palmitoylation inhibitor 2-bromopalmitic acid (2-BP; Sigma-Aldrich, 21604) was added at a final concentration of 100 μM, a concentration known to block ookinete morphogenesis when the drug is mixed in the conversion assay at 1 h after gametocyte activation in ookinete medium (24). Alternatively, the same concentration of 2-BP was added at time zero of the conversion assay.

For the drug screenings, the 400 compounds from the Pathogen Box (MMV) were initially diluted in ookinete medium to a final concentration of 10 μM. The conversion assays were performed as described, and the luciferase activity was measured after 24 h. A second round of screening tested the best hits (>95% inhibition of conversion) at a final concentration of 1 μM. The IC_{50} was determined for the best compounds (>95% inhibition of conversion). For IC_{50} determination, the compounds were initially diluted to a final concentration of 1 μM and serially diluted by a 2-fold factor until no inhibition of conversion was observed. The curves of inhibition and IC_{50} determination were made using GraphPad Prism version 6.0.

Statistical and comparative analysis. Student *t* test analysis was used to calculate statistical differences between sample groups. The Z-factor for the high-throughput assay was calculated as described previously (25), with positive controls being wells with conversion assays as described in Fig. 1b and negative controls being wells with conversion assays with nonparasitized blood or wells with nonactivated *Ookluc* (in both cases, the RLU signal is the same). Previously assessed compound activity

against *Plasmodium* was downloaded (https://chembl.gitbook.io/chembl-ntd/; dataset 21; MMV Pathogen Box).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01053-18.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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