Identification of MMV Malaria Box Inhibitors of

*Plasmodium falciparum* Early-Stage Gametocytes, Using a
Luciferase-based High-Throughput Assay

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Running Head: A HTS Assay for *P. falciparum* Early-Stage Gametocytes

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ABSTRACT

The design of new antimalarial combinations to treat *Plasmodium falciparum* infections requires drugs that, in addition to resolving disease symptoms caused by asexual blood stage parasites, can also interrupt transmission to the mosquito vector. Gametocytes, which are essential for transmission, develop as sexual blood stage parasites in the human host over 8–12 days and are the most accessible developmental stage for transmission-blocking drugs. Considerable effort is currently being devoted to identifying compounds active against mature gametocytes. However, investigations on the drug sensitivity of developing gametocytes, as well as screening methods for identifying inhibitors of early gametocytogenesis, remain scarce. We have developed a luciferase-based high-throughput screening (HTS) assay using tightly synchronous stage I - III gametocytes from a recombinant *P. falciparum* line expressing GFP-luciferase. The assay has been used to evaluate the early stage gametocytocidal activity of the MMV Malaria Box, a collection of 400 compounds with known antimalarial (asexual blood stage) activity. Screening this collection against early-stage (I-III) gametocytes yielded 64 gametocytocidal compounds with IC$_{50}$ values below 2.5 µM. This assay is reproducible and suitable for the screening of large compound libraries, with an average % coefficient of variance (CV) ≤ 5%, an average signal to noise ratio (S:N) > 30 and a Z’ ~0.8. Our findings highlight the need for screening efforts directed specifically against early gametocytogenesis, and indicate the importance of experimental verification of early stage gametocytocidal activity in the development of new antimalarial candidates for combination therapy.
INTRODUCTION

*Plasmodium falciparum* malaria remains a primary global cause of death and disability from infectious disease, particularly in infants and pregnant women (1). Malaria treatment currently relies on artemisinin-based combination therapy (ACT), however, emerging resistance to artemisinins in the field (2, 3) underscores the need to progress new antimalarial candidates through the drug development pipeline. Recent *in vitro* high throughput screening (HTS) campaigns against *P. falciparum* asexual blood stages, the forms responsible for the clinical manifestations of the disease, have identified a wealth of active chemical classes, representing a promising starting point for the discovery of new therapeutic agents (4-7). The current malaria elimination strategy has also highlighted the need for all new antimalarial combination therapies to include components capable of interrupting the transmission of sexual stage gametocytes to *Anopheles* mosquitoes (8, 9). Methods to enable prioritization of inhibitors of the asexual parasite stages based on their additional transmission-blocking activity are therefore urgently required.

*P. falciparum* gametocytes develop through five morphologically distinct stages in the human blood over 8-12 days (10), and thereafter persist in the peripheral blood for weeks (11). They currently represent the most ‘druggable’ transmission-related forms of the parasite. Several *in vitro* methods for assessing the activity of compounds against gametocytes have been published (12). These have been based on measurement of metabolic activity (13, 14), fluorescence intensity from transgenic parasite lines expressing a reporter gene (15) and, more recently, parasite lactate dehydrogenase (LDH) activity (16). Another gametocytocidal assay based on the use of the viability indicator AlamarBlue has been recently shown to be suitable for
HTS in a proof of concept study (17). These methods have focused on the later steps of gametocytogenesis and/or on mature gametocytes, as the immature stages have often been hypothesized to be sensitive to most antimalarial drugs, hence of limited concern. However, an extensive assessment of the degree of sensitivity of early stage gametocytes to antimalarial compounds has never been carried out and the few available studies have been limited to a small number of established drugs (15, 18-22). Furthermore, several early and recent reports suggest that differences exist in the respective chemo-sensitivity of developing gametocytes and asexual stages to antimalarial compounds such as chloroquine (21), pyrimethamine (21), atovaquone (15, 18, 22) and methylene blue (15).

Here we describe a HTS assay for the assessment of chemical compound activity on the early (stage I-III) *P. falciparum* gametocytes, using a luciferase-based approach. We demonstrate its application with reference antimalarial drugs, and report new candidates from the Medicines for Malaria Venture (MMV) Malaria Box, a collection of 400 compounds with known activity against the asexual stages of *P. falciparum* (23).

**MATERIALS AND METHODS**

**Asexual stage cultures.** The *Plasmodium falciparum* transgenic line NF54<sup>Pfs16</sup> and the non-genetically modified strain 3D7 were cultured using O<sup>+</sup> human red blood cells (RBCs) at 5% hematocrit (hct) in parasite culture medium (RPMI 1640 supplemented with 25 mM HEPES (Gibco), 5% AB<sup>+</sup> human male serum (Sigma), 2.5 mg/ml ALBUMAX II (Gibco), 0.37 mM hypoxanthine (Sigma). Culturing medium used for the NF54<sup>Pfs16</sup> line also contained 5 µM blasticidin S (Invitrogen). Cultures were
incubated under standard conditions of 37°C in a gas mixture of 5% O₂, 5% CO₂, 90% N₂. Asexual parasitemia was maintained at approximately 2% by regular dilution of cultures using uninfected RBCs (Fig. 1A). Five days before the induction of gametocytogenesis the NF54<sup>Pfs16</sup> parasites were synchronized at the ring stage by two rounds of exposure to 5% D-sorbitol for 10 minutes, performed 8 hr apart (24) (Fig. 1B).

**Induction and purification of early stage gametocytes.** The approach used to stimulate gametocytogenesis was based on the method first described by Fivelman and colleagues (25), modified to increase yield. To maximize the stage homogeneity of the parasites undergoing the gametocytogenesis induction process, 24 hr before the application of stress (on day -3 of gametocytogenesis, five days after the first sorbitol synchronization) trophozoite cultures were magnetically purified using a MACS<sup>®</sup> column (Miltenyi Biotech) (26). The column-retained trophozoites were eluted and resuspended in fresh RBCs to a final parasitemia of 3% and a decreased hct of 1.25% (Fig. 1C). The cultures were gently shaken overnight to minimize the number of multiply-infected RBCs. The following day generally yielded a high ring parasitemia (average 9.95% ± SEM 0.23%; n = 13). Cultures then received a partial medium change consisting of 3/4 of the total culture volume (day -2, Fig. 1D), and the hct was increased to 2.5%. After a further 24 hr incubation, the stressed parasites at the trophozoite stage were allowed a full medium change and the parasitemia was reduced to 3% (day -1, Fig. 1E). Cultures were incubated overnight on a shaker. The ring stage cultures obtained the following day (day 0 of gametocytogenesis), which included gametocytes beginning to differentiate, were then separated from all other parasite stages by an additional round of magnetic
purification. This purification step removed the later stage gametocytes that had formed spontaneously before the induction process, as well as asexual trophozoites and schizonts that had lost synchronicity, thus enriching for very early stage gametocytes resulting from the standardized induction method only. The flow-through fraction typically yielded ~12% parasitemia (average 11.94% ± SEM 0.33%; n = 10). The cultures were resuspended at 1.25% hct into blastcidin-free malaria culture medium supplemented with 0.5 g/L glucose, as well as 50 mM N-acetyl-D-glucosamine (NAG) to prevent reinvasion of asexual parasites (27) (Fig. 1F), and were incubated overnight.

**Assay development and optimization.** On day one of gametocytogenesis the medium was replaced and the hct level was adjusted according to experimental requirements. The parasites, typically at 10% parasitemia (average 9.96% ± SEM 0.72%; n = 10) were plated without further RBC addition to a final volume of 50 μL per well in sterile 384-well white plates (CulturPlate®, PerkinElmer) using a Multidrop (Thermo Scientific) reagent dispenser under sterile conditions. The plates were then barcoded as appropriate, sealed with gas-exchange membranes (Breathe-Easy®, Sigma-Aldrich) and incubated at standard conditions for 72 hr (Fig. 1G). The luminescence activity was measured on day 4 of gametocytogenesis (Fig. 1H), using the Steadylite plus™ (PerkinElmer) luminescence reporter gene assay system on a MicroBeta® Trilux multidetector luminometer.

The effect of hct level and gametocyte numbers on the luciferase signal was measured to determine the optimal conditions for a linear response. On day one of gametocytogenesis, two independent cultures were plated at 0.17%, 0.46% and
1.25% hct values at their original parasitemia and after a 1:8 dilution with uninfected RBCs. Three more cultures were kept at 0.5% hct and serially diluted (1: 2.6) five times using uninfected RBC. All cultures were plated in 12 replicate wells. After the incubation, 20 µL per well of Steadylite was added and the luminescence signal from the gametocyte density and hct range plates was measured following 1 and 4 hr incubations at room temperature, respectively.

DMSO tolerance was assessed on gametocyte cultures plated into five groups of 24 wells at 0.5% hct in 8 increasing concentrations of DMSO (0.1% to 6.4%), on day 1 of gametocytogenesis. At the end of the incubation, 20 µL per well of Steadylite was added and the luminescence signal was measured after a one-hour incubation at room temperature. The experiments were repeated 2 to 6 times.

To compare the intensity of the luminescence signal from gametocytes to the background signal, two independent gametocyte cultures on day one of gametocytogenesis were plated at 0.5% hct into two groups of 24 replicate wells containing either 0.4% DMSO (negative control, maximum signal) or 2 µM puromycin (positive control, background signal). At the end of the incubation, 20 µL of Steadylite was added and the luminescence signal was measured at three different time points, namely 15 minutes, 2.5 hr and 5 hr after the addition of the luciferase substrate. The average maximum and background signals, as well as the signal-to-noise ratio, were calculated for each time point.

**Final assay conditions.** Experimental compounds were added to the plates at various concentrations, typically in a volume of 5 µL and a final DMSO concentration of 0.4%. Two full columns of each plate were used as in-plate controls, and
contained 0.4% DMSO (negative control, 0% inhibition) and 5 μM puromycin (positive control, 100% inhibition), respectively. Gametocyte cultures were then added to the compound-containing plates at 0.5% hct in a volume of 45 μL per well on day one of gametocytogenesis, and sealed with gas-exchange membranes as described above. At the end of the 72 hr incubation, on day 4 of gametocytogenesis, 25 μL of medium was removed from each well and replaced with 15 μL of Steadylite, using a Biomek® FX high throughput liquid handling system (Beckman Coulter). The plates were light protected and incubated at room temperature for 60 minutes to allow for complete cell lysis, after which time the luminescence signal was measured. Giemsa smears were taken from parallel gametocyte cultures, maintained in similar conditions, to determine the percentage gametocytemia and stage composition.

The assay quality was evaluated by the analysis of the luminescence signal from pairs of full negative (0.4% DMSO) and positive (5 μM puromycin) control plates (n = 4), as well as plates containing a range of 16 concentrations (0.4 nM to 40 μM) of puromycin and dihydroartemisinin (n = 2). Plates were read at regular time points between 40 minutes and 7.5 hr after the addition of Steadylite. The standard assay parameter Z’ from the control plates, as well as the IC$_{50}$ values of each compound were calculated for each time point. The activity of puromycin on gametocytes obtained with the assay was also compared with microscopy-based manual counting of Giemsa smears obtained from different wells of the same dose-range plates (n = 3).
Assay validation. A panel of 23 existing antimalarial drugs, plus puromycin and cycloheximide as controls, was tested for gametocytocidal activity. Stock solutions in 100% DMSO at 10 mM concentration were serially diluted to a range of 21 concentrations, from 0.01 nM to 40 μM, in a final DMSO concentration of 0.4%. These known antimalarials were also tested in parallel against 3D7 and NF54\textsuperscript{Pfs16} asexual stages, using our imaging-based HTS assay (28). Briefly, sorbitol-synchronized P. falciparum 3D7 and NF54\textsuperscript{Pfs16} ring stage cultures were incubated at 2% parasitemia and 0.3% hct in sterile, 384-well clear bottom imaging plates (CellCarrier, PerkinElmer) with compounds for 72 hr at 37°C, 5% CO\textsubscript{2}. At the end of the incubation, parasites were permeabilized using 0.01% Triton-X100, 0.001% saponin, 5 mM EDTA, and stained with 0.5 μg/mL 4',6-diamidino-2-phenylindole (DAPI). Plates were kept light protected at room temperature overnight before being read on an Opera confocal microplate imaging system (PerkinElmer) using the following settings: image size 0.430×0.345 mm\textsuperscript{2}, 20X water objective with a 405-nm excitation line and 420–490-nm band pass emission filter, laser power of 1,870 μW, and camera exposure time of 80 msec. The experiment was carried out on two independent cultures, in duplicate point.

Screening of the MMV Malaria Box and hit confirmation. The MMV Malaria Box (23), comprised of 400 P. falciparum asexual stage inhibitors resulting from recent HTS campaigns of large chemical libraries (5, 7, 29) was screened using our early stage gametocytocidal assay. The library, supplied as five 96-well plates containing 10 mM stock solutions in 100% DMSO, was reformatted into two 384-well plates. Compounds were then diluted 1:8 in 100% DMSO then 1:25 in water, to obtain 50 μM solutions in 4% DMSO. The diluted compounds were dispensed into the assay
plates, as described above, to give a final assay compound concentration of 5 µM. The first two columns in each plate were used for the in-plate negative (0.4% DMSO) and positive (5 µM puromycin) controls.

Primary screening was performed in 3 doses (5, 2.5 and 0.5 µM) and hits were defined as compounds inhibiting early gametocyte development by ≥50% at the highest dose. The hits were cherry-picked from the source plates, serially diluted as described above and tested in 16-point dose-response format, at a concentration range of 0.05 nM – 5 µM.

Eleven exemplar compounds from the MMV Malaria Box were then retested in parallel on NF54<sup>Pfs16</sup> gametocytes and on 3D7 and NF54<sup>Pfs16</sup> asexual stages as described above (n = 2, single point), to compare their respective activities. The experiment was carried out on a separate occasion using a new batch of the MMV Malaria Box. The results were related to the asexual activity data made available by MMV for the Malaria Box compounds, which consist of two <i>P. falciparum</i> 3D7 datasets (23), one generated from our laboratory using the same imaging approach employed in this current study (the “Avery” dataset) (28), and the other derived from ChEMBL (the “ChEMBL” dataset). In addition, to check whether the measured inhibitory activity might be due to a direct luciferase enzymatic inhibition or quenching by the compounds, we also carried out an “artefact” test. For this a single gametocyte culture plate was incubated without treatment for 72 hr in standard conditions. The selected compounds and the luciferase substrate were then added to the plate, and the luciferase activity was measured 1.5 hr later, without extended compound exposure.
**Statistical analysis.** All luminescence data were normalized for detector-dependent intensity differences and analyzed using Microsoft Excel 2010 and SPSS v.19. IC\(_{50}\) values were calculated by a four-parameter non-linear regression fit, and IC\(_{50}\) data were compared by means of Student’s t test or, if normality was not satisfied, a nonparametric Mann-Whitney test, using GraphPad Prism v.5.

**RESULTS**

Our method for gametocytogenesis induction and purification of early stage gametocytes yielded gametocytemia values of 1.3 – 4.0% (mean ± SEM 2.44% ± 0.36%; \(n = 7\) independent inductions) on day 4 of gametocytogenesis. During its 72 h incubation, the assay covered the development of gametocytes from stage I to stage IIb/III, as demonstrated by the stage composition of cultures \((n = 7)\) measured at the end of the assay (day 4 of gametocytogenesis). These mainly consisted of mainly stage III gametocytes (mean ± SEM 72.3% ± 5.1%), and a lesser proportion of stage IIb gametocytes (25.1% ± 5.6%). The only later stages observed were stage IV gametocytes, detected in approximately half of the cultures at very low percentages (4.3% ± 0.8% of the total gametocytes in the cultures positive for stage IV gametocytes).

**Assay development and optimization.** Variations in the hct and gametocytemia levels were explored to assess their effects on the luciferase signal and to determine the optimal values for the assay. A hct increase from 0.2% to 0.5% resulted in a mean ± SD increase of 2.10 ± 0.18 fold in the luminescence signal at every gametocytemia level tested \((\text{ANOVA } p < 0.001; n = 4)\), due to the increased number
of gametocytes in the same volume of culture. A further increase of hct to 1.25%, while also resulting in an increased luminescence signal at low gametocytemia levels (2.30 ± 0.42 fold increase; n = 2) did not provide readout improvements at gametocytemia levels of 2.6% or above (1.03 ± 0.14 fold change; n = 2; Fig. 2A). As these measurements were carried out after a long incubation time following luciferase kit addition (4 hr), it is unlikely that the observed saturation was caused by incomplete cell lysis at high hct values. Given the saturation effect observed at higher values, a hct level of 0.5% was chosen for the assay setup. The choice was further justified by the fact that a strong linear correlation existed between the luminescence signal and gametocyte numbers at each hct level tested, in particular R² = 0.99 for all three independent cultures at 0.5% hct (Fig. 2B). This demonstrates the reliability of this readout system to obtain an accurate estimation of gametocyte numbers in the samples.

Next, the threshold of parasite tolerance to DMSO, the solvent used to dilute most test compounds, was determined. Early stage gametocytes were found to tolerate DMSO up to 0.8%, a concentration that did not reduce the intensity of the luminescence signal compared to DMSO-free controls after a 72 hour incubation (t test p = 0.787; n = 4; Fig. 3). To remain well below this threshold, a final DMSO concentration of 0.4% was adopted for subsequent experiments and screening activities.

The quality of the luciferase signal was assessed by calculating the signal to noise ratio (S:N) (30), based on the measurement of the luciferase activity of gametocytes exposed to 0.4% DMSO (0% gametocyte inhibition, maximum signal) and 5 µM
puromycin (100% inhibition, background noise; Fig. 4). Both the signal and the background were shown to remain relatively stable over 5 hr after the addition of the Steadylite kit. The average signals from DMSO- and puromycin-treated gametocytes from both cultures showed a similar trend, with an increase at 2.5 hr most likely due to the completion of cell lysis, followed by a slight decrease due to the slow luciferase signal decay (Fig. 4). The S:N of the two cultures remained in the same range for the entire duration of the experiment: 64.3 and 47.3, respectively, at 15 min, and 39.7 and 43.9 respectively, 5 hr after the kit addition.

The suitability of the assay for HTS was determined using the Z’ factor, a dimensionless statistical parameter combining the difference between maximal signal and background, and their respective variations (30). The Z’ factor can range between \(-\infty\) and 1, and a value \(\geq 0.5\) generally indicates a good assay (30). At gametocytemia values above 0.1%, the Z’ factor remained consistently above 0.7 (Fig. 5), whereas lower parasite densities resulted in less predictable Z’ values. Taken together, the DMSO tolerance, S:N and Z’ indicated good assay performance and predicted useful flexibility regarding the initial gametocytemia that can be used in the assay.

To ascertain whether the luciferase assay response reflected the actual cell viability in the samples, a comparison between the luciferase readout and manual gametocyte counting by microscopy was performed using the same puromycin treated gametocyte culture. The half inhibitory concentration (IC\(_{50}\)) values of 59.7 nM (95% confidence interval 56.2 – 63.4 nM) and 93.9 nM (95% confidence interval 53.2 – 165.7 nM) obtained using the luciferase approach and manual counting via
microscopy, respectively, showed good agreement between the two methods (Fig. 6). Not surprisingly, the luciferase assay displayed much less variability than manual counting of Giemsa smears, given the subjectivity and lower precision of the latter method.

HTS campaigns often involve large numbers of plates simultaneously prepared throughout the various stages of the assay (e.g. fixation/lysis, placement on stacker at room temperature etc.), however it is not uncommon for the final reading to be undertaken over a period of many hours. For luciferase HTS assays it is therefore critical to determine the time interval between the addition of the luciferase kit / lysis of the cells and the actual reading, that still allows a reliable luciferase measurement. In this study the consistency of the IC$_{50}$ values over time was determined using puromycin and dihydroartemisinin, which showed IC$_{50}$ values of 86.1 ± 3.2 nM and 3.1 ± 0.3 nM, respectively, at the first reading carried out 44 - 50 minutes after kit addition. Fig. 7 shows the extended window of time available for the final assay reading after the luciferase kit was added to the plates. The IC$_{50}$ values obtained by reading the same plates multiple times over a period of 7.5 hr did not show statistically significant differences (ANOVA $p = 0.218$ and 0.996 for puromycin and dihydroartemisinin, respectively). The Z’ values calculated from the in-plate negative (0.4% DMSO) and positive (5 µM puromycin) control wells remained in the 0.7 - 0.8 range throughout the repeated readings (Fig. 7). The reliable and stable determination of gametocytocidal compound potencies demonstrates that our early stage gametocyte assay was suitable for the high-throughput interrogation of large compound libraries.
Assay validation. The inhibitory activity of a panel of 23 antimalarial drugs was assessed on stage I-III NF54\textsuperscript{Pfs16} gametocytes, as well as NF54\textsuperscript{Pfs16} and 3D7 asexual stages (Table 1). The compounds tested exerted a remarkably similar inhibitory activity on the asexual stages of the two related parasite lines. The mean ± SEM IC\textsubscript{50} ratio for NF54\textsuperscript{Pfs16} / 3D7 for the compounds corresponded to 1.43 ± 0.09. In striking contrast, stage I-III NF54\textsuperscript{Pfs16} gametocytes were completely insensitive to three compounds, and showed more than a 5-fold reduced sensitivity than the asexual stages of both parasite lines to eight other compounds in our parallel tests. The observed differences appeared to be dependent on the chemical class of the drugs. Endoperoxides (artemisinin, artemisone, artemether, artesunate and artemimol) and aminoalcohols (mefloquine, lumefantrine, halofantrine, but not quinine) were equally effective on the two parasite forms, while the other classes showed a generally weaker gametocytocidal activity.

MMV Malaria Box screening. The early stage gametocytocidal assay was then used to screen the MMV Malaria Box (23). The ‘biased’ nature of this chemical library, consisting of 400 known \textit{P. falciparum} asexual stage actives, was anticipated to affect the statistical distribution of the screening data, with an expectation of high numbers of compounds affecting early stage gametocytes. Indeed, the activity distribution at the highest dose (5 µM) deviated strongly from normality (Fig. 8A, 8B). To obtain a statistically appropriate hit cutoff, the luciferase signal from 9 negative control plates (0.4% DMSO) and 2 positive control plates (5 µM puromycin) was measured. The data from the control plates (3456 data points) showed a clearly normal distribution (Fig. 8C, 8D), with median % inhibition of 0.58% and median
absolute deviation (MAD) of 3.14%. A corresponding robust hit cutoff (median % inhibition + 3 \times MAD) of 10% inhibition was obtained. However, having verified the statistical boundaries for a reliable cutoff value, we selected only compounds with activity \( \geq 50\% \), as compounds exhibiting less activity at 5 µM are unlikely to be attractive for progression.

The primary screening of the MMV Malaria Box revealed that only 135 of the 400 compounds, i.e. \( \sim \frac{1}{3} \) of the whole library, inhibited early gametocytogenesis by \( \geq 50\% \) at the highest concentration of 5 µM (Table 2). Of these, 87 compounds (65% of the hits) inhibited early gametocytes \( \geq 80\% \). The hit rate dropped to 20% at 2.5 µM and to just above 2% at the 0.5 µM screening dose. The 135 hits obtained at the 5 µM screening dose included 58 drug-like and 77 probe-like molecules. The compounds were cherry-picked and tested in dose response (20 nM to 5 µM) to determine accurate IC\(_{50}\) values. Sixty-four compounds, consisting of 26 drug-like compounds and 38 probe-like compounds produced statistically valid dose-response curves (Fig. 9; Supplementary Table S1). The inhibitory activity of the remaining compounds, mostly low-active hits, did not plateau at the highest concentrations, therefore the 4-parameter dose-response curve did not fit and valid IC\(_{50}\) values could not be determined. Seven drug-like and 13 probe-like compounds showed gametocytocidal activity with submicromolar IC\(_{50}\) values. The five most active compounds of the library, specifically four drug-like compounds and one probe-like compound, showed early stage gametocytocidal IC\(_{50}\) values around 200 nM (Table 3).
With one exception, the most active early stage gametocytocidal compounds were from a subset of the 80 most potent asexual stage inhibitors (plate A) of the MMV Malaria Box (23). These five compounds were retested in parallel on gametocytes and on 3D7 and NF54\textsuperscript{Pfs16} asexual stages on a separate occasion and using a different batch of the MMV Malaria Box, to compare their gametocytocidal activity with their activity on asexual stages. Six more compounds from the MMV Malaria Box (four drug-like and two probe-like), which had shown no gametocytocidal activity on the first screening, were also included in this confirmation experiment to act as asexual stage selective controls (Table 4).

Ten out of the 11 compounds showed excellent agreement in their activity on 3D7 between our test and both publicly available asexual stage datasets. Moreover, the activity on NF54\textsuperscript{Pfs16} asexual stages was found to overlap with the reference asexual data and our 3D7 data for seven compounds of the selected set. Among the remaining four compounds, the drug-like GNF-Pf-3245 showed a greatly reduced activity on all the cultures tested, as opposed to its 220 nM gametocytocidal activity from the screening and the reported asexual data, possibly due to compound degradation during storage / handling. The compounds SJ000091710, GNF-Pf-4188 and GNF-Pf-3159 exhibited weaker inhibition on the NF54\textsuperscript{Pfs16} line in respect to 3D7, a finding that could possibly reflect a reduced sensitivity of the recombinant line to the compounds.

As with our reference drug set, the selected MMV Malaria Box compounds showed differing inhibition profiles on gametocytes and asexual stages (Table 4).
The probe-like compound TCMDC-125554 showed similar inhibitory activity on asexual stages and gametocytes, with IC$_{50}$ values of 81 nM, 265 nM and 174-197 nM against 3D7, NF54$^{Pfs16}$ (asexual blood stages) and NF54$^{Pfs16}$ gametocytes. The quinine analogue GNF-Pf-5561 showed a 9-fold reduced activity against 3D7, and a smaller 4-fold difference against NF54$^{Pfs16}$ asexual stages. A similar trend was observed for TCMDC-125233, a chloroquine analogue which inhibited early stage gametocytes with a 7 times higher IC$_{50}$ than 3D7, and just 3 times higher in respect to NF54$^{Pfs16}$ asexual blood stages. GNF-Pf-5623, another 4-aminoquinoline was clearly less effective against gametocytes, with an 11.2- and 5.5-fold lower activity as compared to 3D7 and NF54$^{Pfs16}$ asexual stages, respectively. The remaining three compounds of the group confirmed their complete selectivity for asexual stages, by being inactive against developing gametocytes at concentrations up to 5 µM, while inhibiting both 3D7 and NF54$^{Pfs16}$ asexual blood stages with IC$_{50}$ values in the range of 23 – 270 nM. It should be noted that two of the compounds which showed a lower activity on asexual NF54$^{Pfs16}$ parasites than on 3D7, namely GNF-Pf-4188 and GNF-Pf-3159, completely failed to inhibit NF54$^{Pfs16}$ gametocytes, suggesting that these compounds are also selective for asexual stages. All the selected compounds showed negligible inhibition values in the range 4% - 11% in the “artefact” test, ruling out possible direct luciferase enzyme inhibition/quenching effect by the compounds.

The 64 confirmed hits obtained from screening the MMV Malaria Box collectively inhibited developing gametocytes with an average IC$_{50}$ ± SEM of 1.09 ± 0.09 µM, significantly higher than their asexual activity in both the “Avery” (IC$_{50}$ = 0.33 ± 0.05 µM) and the “ChEMBL” (IC$_{50}$ = 0.41 ± 0.08 µM) datasets (nonparametric Mann-
Whitney test $p < 0.0001$), while no difference emerged from the comparison of the two reference asexual datasets (nonparametric Mann-Whitney test $p = 0.1686$; Fig. 10). Confirmed gametocytocidal hits with an asexual IC$_{50} \leq 1$ µM against 3D7 were 56 (‘Avery’ dataset) or 51 (‘ChEMBL’ dataset), while only 20 of the hits showed an early stage gametocytocidal potency below this threshold in our assay. Although some of the hits could have a lower impact on the NF54$^{Pfs16}$ parasite line than on 3D7, as observed for 3 of the 10 representative exemplars of the MMV Malaria Box, this preliminary comparison suggests that the hits may have less potent activity on developing gametocytes than asexual stages.

The described HTS assay is currently being utilized to screen larger, unbiased compound libraries against early stage gametocytes. Fig. 11 shows a summary of the assay parameters from 106 plates screened to date, corresponding to more than 40,000 data points, resulting from 5 separate screening campaigns involving 10 independent gametocyte inductions and culturing rounds. The assay has demonstrated excellent performance and reproducibility, with average %CV $\leq 5\%$, S:N consistently above 30 and $Z'$ values approximating 0.8 per campaign.

**DISCUSSION**

In this report we described the first high-throughput assay utilized to test chemical compounds on synchronized cultures of developing *P. falciparum* gametocytes. To obtain populations of early stage gametocytes resulting from the commitment of a single generation of parasites, we first maximized the synchronicity of the asexual stage cultures prior to the sexual differentiation induction process, by using 5% D-sorbitol synchronization (24) and magnetic purification (26). Then we removed the
“contaminating” existing gametocytes from newly induced cultures, using magnetic purification. Sexual differentiation was triggered by applying a combination of stress factors known to stimulate gametocytogenesis in vitro, namely a drop in hct, a high parasitemia and the consequent hemolysis (31) due to schizont rupture, as well as nutritional stress (32, 33). Luciferase-based approaches have previously been shown to be useful for the development of high-throughput assays for compound screening against the asexual stages of *P. falciparum* (34-36). Our HTS assay for early gametocytogenesis was developed using the *P. falciparum* transgenic line NF54\textsuperscript{Pfs16}. This parasite expresses a GFP-luciferase fusion reporter gene under the control of the promoter of the gametocytogenesis-specific gene *Pfs16* (18). The expression of *Pfs16* starts in sexually committed schizonts, and persists for the duration of gametocyte development (37, 38). The NF54\textsuperscript{Pfs16} luciferase activity peaks on day 2 of gametocytogenesis and gradually declines. We performed the assay readout on day 4, when the parasite luciferase activity is at ~80% of the day two value (18), a level that still ensures an excellent signal window. In our assay, gametocytes were exposed to compounds from day 1 (stage I) up until day 4 (stage IIb-III) of gametocytogenesis. It is possible that compounds exerting their maximal inhibitory effect on ring stage parasites might prove more effective on gametocytes than in our study, if day 0 gametocytes at the ring stage (pre-stage I), were exposed, as in the early stage gametocytocidal assay by Buchholz *et al.* (15).

The expected practical usefulness of compounds whose efficacy is restricted to the ring stage gametocyte is limited, given the short time window available for suppression over the long total gametocyte developmental time. Therefore our approach of using day 1 – 4 of gametocytogenesis for the determination of
gametocytocidal activity is expected to allow the detection of longer acting compounds, with possibly higher gametocyte-specificity, and is in keeping with the WHO recommendations to treat patients with 3-day courses of antimalarial combination therapies.

Immature gametocytes have often been considered susceptible to drugs acting on asexual development stages. However, studies that have directly tested the susceptibility of early stage (I – III) gametocytes to antimalarial compounds and drugs are scarce, and where reported, the authors have investigated relatively few compounds (15, 18-22). To the best of our knowledge, our evaluation of 23 reference antimalarial drugs and the screening of the MMV Malaria Box (400 compounds active against asexual blood stages) using the luciferase-based assay described here represents the largest investigation of the chemo-sensitivity of *P. falciparum* early gametocytogenesis. If a similar chemo-sensitivity profile of early stage gametocytes and asexual stages is assumed, the hit rate of 34% obtained in our MMV Malaria Box screening at the highest screening dose or 5 µM is a surprisingly low proportion, considering that the Malaria Box constitutes a set of known asexual blood stage inhibitors (23). Moreover, the overall gametocytocidal potency of the best 64 MMV Malaria Box hits appears lower than the asexual stage potency reported in the publicly available asexual data for *P. falciparum* 3D7. It is important to note that our parallel tests with 3D7 and NF54<sup>Pfs16</sup> asexual stages (Tables 1 and 4) utilized the same assay approach previously used by our lab to generate the asexual stage data of the MMV Malaria Box (the ‘Avery’ dataset) (28). These data allowed us to confirm that the two parasite lines (NF54<sup>Pfs16</sup> - generated in NF54, and 3D7 - a clone of NF54) have similar asexual chemo-sensitivities, with
only 3 out of 33 total compounds compared showing a difference. Furthermore, the activity of the 10 valid MMV Malaria Box exemplars against 3D7 asexual blood stage parasites was in good agreement with both the ‘Avery’ and the ‘ChemBL’ datasets. This provided sufficient justification for an overall comparison of the gametocytocidal hit potencies with the reference 3D7 asexual data. Collectively, our results demonstrate that it cannot be assumed that asexual blood stage activity predicts potency against developing gametocytes. As a consequence, compound screening on developing gametocytes should be included in the current efforts to identify transmission-blocking compounds, and adequate attention should be paid to the early stage gametocytocidal activity of new antimalarial drug candidates being developed. Developing gametocytes share some important features with their asexual counterparts, such as hemoglobin digestion and heme detoxification through the formation of hemozoin (39), which is considered to be the target process of 4-aminoquinoline antimalarials (40). Increasing evidence is nonetheless accumulating on the differences between immature gametocytes and asexual blood stages, such as their distinct mitochondrial metabolism (41, 42) and their unique sequestration mechanisms (43, 44). The reduced sensitivity of these forms to many antimalarial compounds warrants further investigation into the underlying causes. A detailed characterization of the chemo-sensitivity of the entire developmental process of P. falciparum gametocytogenesis will provide useful information for the development of effective transmission-blocking drugs and increase our understanding of gametocyte biology (45, 46). The compound activity data of well known drugs and new candidates generated in our study allows for the selection of useful chemical tools for such studies.
Three out of the five most potent gametocytocidal compounds identified by our pilot screening of the MMV Malaria Box are structurally related to chloroquine. This raises some concerns about the chances of identifying potent gametocytocidal drugs with novel mechanism of action from focused libraries such as the MMV Malaria Box, especially considering that the rationale adopted for the selection of compounds to be included into this collection was to maximize chemical diversity (23) and hence potentially target diversity. The comparison of our results with the outcomes from further screenings of the MMV Malaria Box, as well as the screening of larger, non-focused libraries, on both early and late stage gametocytes will be of great interest to investigate these issues.

As with any in vitro screening report, the compound activity data produced in this study will benefit from additional studies to confirm activity, as confounding factors may exist. A general limitation of in vitro studies is that compounds requiring metabolic activation in the host, such as primaquine (47), are likely to be overlooked. The use of different technologies, namely luciferase and imaging, for gametocytes and asexual stages, respectively, has to be taken into account for potential biases. The impact of these different approaches on the estimated inhibitory potencies, however, is not likely to have affected the results to such a degree as to account for the large discrepancies in sensitivity we observed between the two different stages of the parasite life cycle. The comparison of the asexual data for 64 of the MMV Malaria Box compounds from the ‘ChEMBL’ and the ‘Avery’ datasets, which were also obtained with different technologies (LDH / total fluorescence and High-Content imaging, respectively), showed no statistical differences. The use of the genetically manipulated parasite line NF54^{Pfs16} involved the risk of generating biased results
due to a different sensitivity to compounds than its parental line. This possibility was investigated by comparing the activity of 23 reference antimalarial drugs and 10 representative exemplars from the MMV Malaria Box on the asexual stages of NF54\(^{Pfs16}\) and 3D7, however a reduced sensitivity of the recombinant line was found for only 3 compounds, thus reducing such concern. Additionally, being based on the expression of a luciferase marker linked to the early gametocytogenesis promoter \(Pfs16\), our assay may detect residual luciferase activity from dying parasites. To minimize this potential limitation, however, we employed a long time of exposure to compounds (72 hr). Orthogonal screenings of the MMV Malaria Box (and other libraries) using complementary technologies such as High-Content Imaging are ongoing in our laboratory, to further validate our results.

Ensuring that new antimalarial combination therapies in the development pipeline eliminate early stage gametocytes along with the asexual stages ensures a reduction of the parasite pool from which mature, transmissible gametocytes can arise, thereby helping to suppress \(P. falciparum\) transmission. A recent theoretical population study suggested that antibodies (or a drug) targeting developing gametocytes could have a higher impact on mature gametocyte density than equally potent antibodies (or a drug) that kill the late stages only, while the latter would have a greater impact on gametocyte carriage duration (48). To measure the impact of different drug sensitivity of developing gametocytes and asexual stages in the field, however, is extremely difficult. The quantification of developing gametocytes in clinical settings is challenging, due to the sequestration of the early sexual forms in the bone marrow (49) and, possibly, the spleen (50), and the measurement of late stage gametocyte density as a delayed estimation of early stage survival is subject to a large number of
confounding factors, such as the direct activity of combination therapies on the late stage gametocytes and pre-existing gametocytemia.

In conclusion, our HTS assay proved reproducible and suitable for the screening of large compound libraries against developing *P. falciparum* gametocytes. Our findings on the activity of existing drugs on early stage gametocytes and the pilot screening of the MMV Malaria Box demonstrate the importance of the experimental confirmation on early stage gametocytocidal activity for all new drugs being developed for combination therapy. We recommend the inclusion of early stage gametocytocidal activity in the desired Target Candidate Profile for malaria transmission-blocking drugs (9), and highlight the necessity of screening efforts directed specifically against early gametocytenesis within the transmission blocking drug discovery strategy. Finally, the investigation of the factors underlying the differential sensitivity profile of developing gametocytes to some antimalarial compound classes will deepen our knowledge of gametocyte biology and will contribute to the development of more effective gametocytocidal drugs.

**ACKNOWLEDGEMENTS**

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development of the assay. The Australian Red Cross Blood Service is gratefully acknowledged for the provision of human erythrocytes.

REFERENCES


Figure 1 – *P. falciparum* culturing (A, B), gametocyte induction (C-F) and assay method (G, H). Hct: hematocrit; troph: trophozoite; NAG: N-acetylglucosamine
Figure 2 – Effect of hematocrit (hct) on luminescence signal of 2 independent cultures at 3.9% and 2.6% day 4 gametocytemia, respectively (A), and luminescence signal from serial dilution of 3 independent cultures at 0.5% hct (B). The luciferase signal was measured after 72 hours of incubation in 384-well plates, on day 4 of gametocytogenesis, 4 hours (A) or 60 minutes (B) after Steadylite addition.
**Figure 3** – Early stage gametocyte tolerance to increasing dimethyl sulfoxide (DMSO) concentrations (average percent of 0% DMSO ± SEM of 2-6 independent cultures, on day 4 of gametocytogenesis, after 72 hours of incubation in 384-well plates with DMSO).
Figure 4 – Average signal from negative (0.4% DMSO, solid bars) and positive control (5 µM puromycin, pattern-filled bars) wells (n = 24) from two independent cultures (left axis), and corresponding signal to noise (S/N) ratio (solid lines, right axis) measured at 3 consecutive time points after the addition of 20 µL Steadylite, on day four of gametocytogenesis.
Figure 5. Effect of gametocytemia level on assay performance. Z' values for increasing gametocytemia were calculated to determine optimal conditions, with a cutoff of 0.5. Each symbol indicates an independent gametocyte culture at 0.5% hematocrit.
Figure 6 – Comparison between luciferase- (filled symbols) and microscopy-based (hollow symbols) measurement of puromycin standard inhibition curve.
Figure 7 – Signal stability after luciferase kit addition. IC$_{50}$ curves (above) and trends of IC$_{50}$ and Z' values over time (below, filled and open symbols, respectively) of puromycin (A, C) and dihydroartemisinin (DHA; B, D). Values were obtained by reading the same plates at different time points after the addition of Steadylite luciferase kit ($n = 2$ independent cultures and 4 technical replicates). Trends of Z' values over time were obtained from in-plate negative (0.4% DMSO) and positive control (5 µM puromycin) wells.
Figure 8 – Activity distribution and Q-Q plot (based on estimates of the quantiles for normal distribution) of screening data with the MMV Malaria Box compounds (average of 3 replicates; n = 400 each @ 5 μM; A, B respectively), and of 0.4% DMSO negative control plates (9 replicates, n = 384 each; C, D respectively).
Figure 9 – Mean IC$_{50}$ values ± SEM of gametocytocidal hits identified from screening the MMV Malaria Box. Data from triplicate cultures.
Figure 10 – *P. falciparum* asexual (3D7) and early-stage (NF54Pfs16) gametocytocidal activity of the 64 confirmed MMV malaria box gametocytocidal hits. Asexual data obtained from reference 28 (Table S1). The dotted line indicates the limit beyond which statistically valid IC\textsubscript{50} values could not be obtained for early stage gametocytes. ** correspond to statistically significant difference with \( p < 0.0001 \)
Figure 11 – Assay parameters for each of 5 screening runs and 10 gametocyte cultures from independent induction processes (A). Exemplar screening run of 20 plates (run no. 3; B). Average luminescence signal (left axis) from 0.4% DMSO wells (positive control, filled symbols, n = 16), and 5 μM puromycin (hollow symbols, n = 16). The continuous line represents the Z’ value of each plate (right axis). N/D = not done.
TABLE 1 Activity of established antimalarial drugs and drug candidates on asexual blood stages and early-stage gametocytes of the indicated *P. falciparum* line.

<table>
<thead>
<tr>
<th>compound</th>
<th>3D7 asexual stages (IC₅₀ nM ± SEM)</th>
<th>NF5₄&lt;sup&gt;Prsb₁₆&lt;/sup&gt; asexual stages (IC₅₀ nM ± SEM)</th>
<th>stage I-III gametocytes (IC₅₀ nM ± SEM)</th>
<th>IC₅₀ ratios&lt;sup&gt;*&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>artemimol</td>
<td>0.24 ± 0.003</td>
<td>0.37 ± 0.002</td>
<td>0.90 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>artemisone</td>
<td>0.18 ± 0.003</td>
<td>0.21 ± 0.02</td>
<td>0.94 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>artemunate</td>
<td>0.82 ± 0.17</td>
<td>1.02 ± 0.12</td>
<td>2.96 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>artemether</td>
<td>1.99 ± 0.18</td>
<td>3.24 ± 0.74</td>
<td>3.73 ± 0.83</td>
<td></td>
</tr>
<tr>
<td>artemisinin</td>
<td>3.87 ± 0.07</td>
<td>5.20 ± 1.16</td>
<td>12.91 ± 3.09</td>
<td></td>
</tr>
<tr>
<td>chloroquine</td>
<td>4.69 ± 0.58</td>
<td>12.07 ± 0.76</td>
<td>76.75 ± 4.78</td>
<td></td>
</tr>
<tr>
<td>amodiaquine</td>
<td>17.90 ± 0.25</td>
<td>24.13 ± 0.39</td>
<td>189.71 ± 24.75</td>
<td></td>
</tr>
<tr>
<td>AQ-13</td>
<td>15.15 ± 0.89</td>
<td>23.29 ± 2.43</td>
<td>221.52 ± 4.68</td>
<td></td>
</tr>
<tr>
<td>naphthoquine</td>
<td>39.75 ± 14.82</td>
<td>46.88 ± 5.76</td>
<td>298.23 ± 58.65</td>
<td></td>
</tr>
<tr>
<td>NPC-1161B</td>
<td>3636.87 ± 1020.05</td>
<td>3378.22 ± 417.58</td>
<td>3396.26 ± 7.17</td>
<td></td>
</tr>
<tr>
<td>tafenoquine</td>
<td>3113.30 ± 917.36</td>
<td>4066.55 ± 56.05</td>
<td>3628.38 ± 86.31</td>
<td></td>
</tr>
<tr>
<td>primaquine</td>
<td>100% inhibition at 40 µM</td>
<td>100% inhibition at 40 µM</td>
<td>80% inhibition at 40 µM</td>
<td></td>
</tr>
<tr>
<td>pyronaridine</td>
<td>19.74 ± 6.26</td>
<td>24.39 ± 1.21</td>
<td>168.09 ± 40.18</td>
<td></td>
</tr>
<tr>
<td>quinine</td>
<td>29.25 ± 2.91</td>
<td>49.89 ± 9.97</td>
<td>374.94 ± 5.62</td>
<td></td>
</tr>
<tr>
<td>mefloquine (+ RS)</td>
<td>30.39 ± 14.29</td>
<td>27.76 ± 1.44</td>
<td>49.72 ± 2.12</td>
<td></td>
</tr>
<tr>
<td>mefloquine (Racemic)</td>
<td>35.68 ± 17.89</td>
<td>22.60 ± 2.95</td>
<td>51.70 ± 4.76</td>
<td></td>
</tr>
<tr>
<td>lumefantrine</td>
<td>17.98 ± 4.66</td>
<td>39.44 ± 5.33</td>
<td>7.82 ± 2.04</td>
<td></td>
</tr>
<tr>
<td>halofantrine</td>
<td>14.96 ± 3.57</td>
<td>27.49 ± 0.40</td>
<td>15.13 ± 4.93</td>
<td></td>
</tr>
<tr>
<td>pentamidine</td>
<td>63.23 ± 15.32</td>
<td>96.89 ± 24.12</td>
<td>1014.88 ± 19.21</td>
<td></td>
</tr>
<tr>
<td>atovaquone</td>
<td>0.27 ± 0.04</td>
<td>0.29 ± 0.02</td>
<td>100% inhibition at 40 µM</td>
<td></td>
</tr>
<tr>
<td>methylene blue</td>
<td>3.44 ± 0.45</td>
<td>3.79 ± 0.05</td>
<td>29.64 ± 0.91</td>
<td></td>
</tr>
<tr>
<td>pyrimethamine</td>
<td>4.35 ± 0.60</td>
<td>8.99 ± 0.75</td>
<td>&gt;40 µM</td>
<td></td>
</tr>
<tr>
<td>clindamycin</td>
<td>7.56 ± 1.06 (75%)&lt;sup&gt;†&lt;/sup&gt;</td>
<td>9.83 ± 0.09 (50%)&lt;sup&gt;†&lt;/sup&gt;</td>
<td>&gt;40 µM</td>
<td></td>
</tr>
<tr>
<td>cycloheximide</td>
<td>45.49 ± 15.49</td>
<td>61.12 ± 0.49</td>
<td>119.50 ± 8.91</td>
<td></td>
</tr>
<tr>
<td>puromycin</td>
<td>61.95 ± 24.51</td>
<td>156.34 ± 54.30</td>
<td>144.55 ± 6.35</td>
<td></td>
</tr>
</tbody>
</table>

<sup>*</sup> white bars = IC₅₀ (asexual NF5₄<sup>Prsb₁₆</sup>) / IC₅₀ (asexual 3D7); grey bars = IC₅₀ (gametocyte NF5₄<sup>Prsb₁₆</sup>) / IC₅₀ (asexual 3D7); black bars = IC₅₀ (gametocyte NF5₄<sup>Prsb₁₆</sup>) / IC₅₀ (asexual NF5₄<sup>Prsb₁₆</sup>); the grey line indicates IC₅₀ ratio = 1 (equal inhibitory activity)

<sup>†</sup> complete inhibition not achieved, the number in brackets indicates the % inhibition at plateau.
TABLE 2 Activity of the MMV malaria box compounds on early stage *P. falciparum* gametocytes.

<table>
<thead>
<tr>
<th>concentration</th>
<th>gametocytocidal hits</th>
<th>inactives</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50% – 79% inhibition</td>
<td>≥ 80% inhibition</td>
</tr>
<tr>
<td>5 μM</td>
<td>48 (12.00%)</td>
<td>87 (21.75%)</td>
</tr>
<tr>
<td>2.5 μM</td>
<td>42 (10.50%)</td>
<td>38 (9.50%)</td>
</tr>
<tr>
<td>0.5 μM</td>
<td>8 (2.00%)</td>
<td>1 (0.25%)</td>
</tr>
</tbody>
</table>
TABLE 3 Early stage gametocytocidal activity of the most active compounds from the Medicines for Malaria Venture (MMV) Malaria Box.

<table>
<thead>
<tr>
<th>Compound Code</th>
<th>IC50 (μM) ± SEM</th>
<th>Hillslope ± SEM</th>
<th>R²</th>
<th>Cytotoxicity</th>
<th>Liver Stage Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHEMBL586906</td>
<td>0.214 ± 0.008</td>
<td>2.540 ± 0.214</td>
<td>0.9873</td>
<td>MRC5 &gt;32 μM; Huh7 = 78 μM</td>
<td>inactive</td>
</tr>
<tr>
<td>CHEMBL602211</td>
<td>0.220 ± 0.010</td>
<td>2.184 ± 0.188</td>
<td>0.9897</td>
<td>Huh7 &gt; 100 μM</td>
<td>inactive</td>
</tr>
<tr>
<td>CHEMBL317364</td>
<td>0.249 ± 0.011</td>
<td>6.431 ± 4.530</td>
<td>0.9668</td>
<td>Huh7 &gt; 30 μM; MRC5 &gt; 32 μM</td>
<td>inactive</td>
</tr>
<tr>
<td>CHEMBL587083</td>
<td>0.252 ± 0.005</td>
<td>6.926 ± 0.970</td>
<td>0.9874</td>
<td>MRC5 &gt; 32 μM</td>
<td>inactive</td>
</tr>
<tr>
<td>CHEMBL533946</td>
<td>0.197 ± 0.007</td>
<td>2.771 ± 0.244</td>
<td>0.9868</td>
<td>MRC5 &gt; 32 μM; HEK293 &gt; 35 μM</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

Data on rule of 5 violations, AlogP, cytotoxicity and liver stage activity obtained from ChEMBL database (https://www.ebi.ac.uk/chembl/malaria/)
<table>
<thead>
<tr>
<th>Compound</th>
<th>Set</th>
<th>3D7 IC\textsubscript{50} 'Avery' dataset (nM) \textsuperscript{a}</th>
<th>3D7 IC\textsubscript{50} 'ChEMBL' dataset (nM)</th>
<th>3D7 IC\textsubscript{50} (nM ± SEM)</th>
<th>NF54\textsubscript{Pfs16} asexual IC\textsubscript{50} (nM ± SEM)</th>
<th>NF54\textsubscript{Pfs16} early gametocytes IC\textsubscript{50} (nM ± SEM)</th>
<th>NF54\textsubscript{Pfs16} early gametocytes IC\textsubscript{50} retest (nM ± SEM)</th>
<th>artefact test (inhibition @ 5 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNF-Pf-5561</td>
<td>Drug</td>
<td>42.3</td>
<td>61.1</td>
<td>21.2 ± 3.3</td>
<td>50.5 ± 6.3</td>
<td>213.7 ± 7.9</td>
<td>202.5 ± 10.1</td>
<td>-0.1%</td>
</tr>
<tr>
<td>GNF-Pf-5623</td>
<td>Drug</td>
<td>58.1</td>
<td>7.2</td>
<td>22.6 ± 3.3</td>
<td>45.9 ± 4.2</td>
<td>249.3 ± 10.9</td>
<td>254.4 ± 10.6</td>
<td>4.0%</td>
</tr>
<tr>
<td>TCMDC-125233</td>
<td>Drug</td>
<td>82.2</td>
<td>62.4</td>
<td>23.3 ± 2.8</td>
<td>57.1 ± 7.9</td>
<td>252.4 ± 5.0</td>
<td>173.7 ± 9.2</td>
<td>-4.5%</td>
</tr>
<tr>
<td>TCMDC-125554</td>
<td>Probe</td>
<td>22.6</td>
<td>278.7</td>
<td>80.6 ± 17.4</td>
<td>264.5 ± 90.7</td>
<td>197.0 ± 7.0</td>
<td>174.3 ± 11.7</td>
<td>6.0%</td>
</tr>
<tr>
<td>TCMDC-124919</td>
<td>Drug</td>
<td>6.7</td>
<td>15.8</td>
<td>23.5 ± 3.1</td>
<td>54.8 ± 8.5</td>
<td>&gt; 5 µM</td>
<td>&gt; 5 µM</td>
<td>-3.0%</td>
</tr>
<tr>
<td>TCMDC-125225</td>
<td>Drug</td>
<td>25.5</td>
<td>87.9</td>
<td>29.0 ± 3.4</td>
<td>83.4 ± 14.8</td>
<td>&gt; 5 µM</td>
<td>&gt; 5 µM</td>
<td>1.2%</td>
</tr>
<tr>
<td>GNF-Pf-4475</td>
<td>Probe</td>
<td>118.0</td>
<td>549.0</td>
<td>120.0 ± 34.2</td>
<td>269.1 ± 47.8</td>
<td>&gt; 5 µM</td>
<td>&gt; 5 µM</td>
<td>11.4%</td>
</tr>
<tr>
<td>SJ000091710</td>
<td>Drug</td>
<td>117.0</td>
<td>61.9</td>
<td>200.7 ± 29.6</td>
<td>50% @ 5 µM</td>
<td>80% @ 5 µM</td>
<td>50% @ 5 µM</td>
<td>3.5%</td>
</tr>
<tr>
<td>GNF-Pf-4188</td>
<td>Drug</td>
<td>350.0</td>
<td>988.4</td>
<td>833.3 ± 193.6</td>
<td>100% @ 5 µM</td>
<td>&gt; 5 µM</td>
<td>&gt; 5 µM</td>
<td>2.8%</td>
</tr>
<tr>
<td>GNF-Pf-3159</td>
<td>Probe</td>
<td>179.0</td>
<td>2464.0</td>
<td>569.7 ± 141.9</td>
<td>100% @ 5 µM</td>
<td>&gt; 5 µM</td>
<td>&gt; 5 µM</td>
<td>-1.6%</td>
</tr>
<tr>
<td>GNF-PF-3245</td>
<td>Drug</td>
<td>683.0</td>
<td>743.0</td>
<td>100% @ 5 µM</td>
<td>75% @ 5 µM</td>
<td>220.0 ± 9.9</td>
<td>1945.4 ± 200.0</td>
<td>0.9%</td>
</tr>
<tr>
<td>dihydroartemisinin</td>
<td>ND</td>
<td>ND</td>
<td>2.1 ± 1.7</td>
<td>7.2 ± 2.1</td>
<td>10.6 ± 1.1</td>
<td>7.5 ± 0.4</td>
<td>-5.4%</td>
<td></td>
</tr>
<tr>
<td>puromycin</td>
<td>ND</td>
<td>ND</td>
<td>61.9 ± 24.5</td>
<td>156.3 ± 54.3</td>
<td>110.9 ± 1.7</td>
<td>145.5 ± 6.3</td>
<td>-2.7%</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} P. falciparum 3D7 asexual stage data obtained from Table S1 of reference 28. ND, not determined.