A chemical susceptibility profile of the *Plasmodium falciparum* transmission stages by complementary cell-based gametocyte assays

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Objectives: As most available antimalarial drugs are ineffective against the *Plasmodium falciparum* transmission stages, new drugs against the parasite's gametocytes are urgently needed to combat malaria globally. The unique biology of gametocytes requires assays that need to be specific, to faithfully monitor anti-gametocyte activity, and to be easy to perform, cheap and scalable to high-throughput screening (HTS).

Methods: We developed an HTS cell-based assay with *P. falciparum* gametocytes specifically expressing a potent luciferase. To confirm HTS hit activity for several parasite genotypes, the luciferase assay and the gametocyte lactate dehydrogenase (LDH) assay, usable on any parasite isolate, were compared by screening antimalarial drugs and determining IC_{50} values of anti-gametocyte hits from the 'Malaria Box' against early- and late-stage gametocytes.

Results: Comparison of the two assays, conducted on the early and on late gametocyte stages, revealed an excellent correlation (R^2 ~ 0.9) for the IC_{50} values obtained by the respective readouts. Differences in susceptibility to drugs and compounds between the two parasite developmental stages were consistently measured in both assays.

Conclusions: This work indicates that the luciferase and gametocyte LDH assays are interchangeable and that their specific advantages can be exploited to design an HTS pipeline leading to new transmission-blocking compounds. Results from these assays consistently defined a gametocyte chemical susceptibility profile, relevant to the planning of future drug discovery strategies.

Introduction

Despite international efforts, malaria still accounts for roughly 600000 deaths per year.[1] A major change in international strategy, from malaria control towards elimination and eradication, has illuminated important gaps in knowledge and tools. In this context, new drugs with the potential to inhibit or completely prevent *Plasmodium* transmission, or which target the hepatic stages, are now being prioritized.[2]

Stage V *Plasmodium* gametocytes are the intraerythrocytic sexual forms taken up by *Anopheles* mosquitoes and are responsible for parasite transmission to the vector. At present, only primaquine can be used *in vivo* as a transmission-blocking drug.[3] However, its toxicity in glucose-6-phosphate dehydrogenase-deficient patients makes the identification of new drugs urgent.

To this end, an intense effort to establish novel screening assays for anti-gametocyte compounds has been made over the past few years.[4] We contributed to this effort by pioneering the detection of the parasite lactate dehydrogenase (pLDH),[5] successfully used in drug screening against asexual parasites,[6] as a reliable marker for *Plasmodium falciparum* gametocyte viability.[7] This assay is robust and usable on any parasite strain, including field isolates. However, the assay requires purity of the gametocyte population, as pLDH is present also in asexual parasites, and a long assay time, as pLDH activity persists for ~24 h after gametocyte death. To achieve specificity for gametocytes, coupled with increased sensitivity and scalability, we exploited the unsurpassed versatility of bioluminescent assays, based on luciferase reporters driven by gametocyte-specific promoters. After the first report of gametocyte assays using *P. falciparum* lines expressing firefly luciferase,[8,9] we introduced red- and green-emitting luciferases from the click beetle *Pyrophorus plagiophthalamus* into malaria parasites under the control of the *pfs16* gametocyte-specific promoter.[10] Importantly, we also introduced

the use of a non-lysing D-luciferin substrate formulation, largely overcoming the drawback of residual reporter signal from unhealthy or dead gametocytes. These improvements are counterbalanced only by the need to use transgenic parasites.

The comparison of gametocyte susceptibility to 10 compounds with four different cell assays suggested the value of screening chemotype diversity with more than a single assay platform.[11]

Here, we report a head-to-head comparison of the pLDH and luciferase-based gametocyte assays. Results of parallel assays on the small compound library called the Validation Set[12] and on hits from the Malaria Box,[13] both from Medicine for Malaria Venture (MMV), showed an excellent correlation between the assays, despite the fact that they measure different biomarkers. More importantly, readouts of both assays positively correlated with the results of standard membrane-feeding assays (SMFAs). This provides a chemical susceptibility profile of parasite sexual blood stages based on independent assays, with good predictability of the transmission-blocking efficacy of compounds. This work indicates that the interchangeability of the pLDH and luciferase assays can be exploited in a high-throughput screening (HTS) pipeline for transmission-blocking compounds to validate compounds against several parasite genotypes.

Materials and methods

P. falciparum culture and gametocyte induction

P. falciparum strains were cultured and gametocytes obtained as described in Supplementary Materials and methods (available in Supplementary data at *JAC* Online).[14] Routinely early- and late-stage gametocytes were exposed to compounds at days 4 and 9, respectively, after *N*-acetylglucosamine (NAG) addition. Percentages of the different gametocyte maturation stages were determined on Giemsa-stained smears.

Plasmid construction and parasite transfection

Details of the production and transfection of the plasmid used to chromosomally integrate the *pfs16*-CBG99 luciferase cassette in the 3D7 fatty acid elongase-1 encoding gene *pfelo1*, dispensable for gametocyte, mosquito and liver stage development (D. A. Fidock, Columbia University, New York, USA, personal communication) are provided in Cevenini *et al*.[10] and in the Supplementary Materials and methods.

Luciferase assay

To establish the gametocyte luciferase (GC-LUC) assay, gametocytes expressing the CBG99 luciferase were used to compare bioluminescence emission kinetics with the commercial Britelite™ kit and with the non-lysing substrate D-luciferin (1 mM in 0.1 M citrate buffer, pH 5.5). Briefly, 100 mL aliquots of gametocyte culture were transferred to 96-well white microplates and 100 mL of each substrate was added. Bioluminescence emissions were acquired for 45 min with 300 – 500 ms integration time using a Synergy4-BioTek reader.

Drug susceptibility assays in 96 and 384 wells

Drugs were stored in stocks in DMSO (see Supplementary data). The primary screening of the MMV Validation Set was performed at 5 mM, in 96-well plates, using 100 nM epoxomicin and 1 mM dihydroartemisinin as positive and 0.5% DMSO as negative untreated control. In dose–response experiments, compounds were prepared by serial dilution, in plate, in complete medium. The concentration range was 7.8 – 1000 nM for dihydroartemisinin, 0.78 – 100 nM for epoxomicin, 1.95 – 20000 nM for the MMV Validation Set compounds and 19.5 – 10000 nM for the Malaria Box.

Gametocyte viability was evaluated by both the GC-LUC and the pLDH assay. In the GC-LUC assay, drug-treated gametocyte samples were transferred to 96-well black microplates and D-luciferin (1 mM in citrate buffer 0.1 M, pH 5.5) was added at a 1:1 volume ratio. Luminescence measurements were performed after 10 min with 500 ms integration time. The pLDH assay was performed as described previously[7] and in the Supplementary data. Results of the MMV Validation Set[12] primary screening were expressed as reported in the Supplementary data.

To scale up the GC-LUC assay to 384-well plates, the sensitivity threshold was established by serially diluting a 1% gametocyte culture using complete medium and red blood cells at 2% haematocrit. Serial dilutions of haematocrit were also tested, maintaining a fixed number of gametocytes. To optimize reading time, bioluminescence emissions were acquired for 30 min with 500 ms integration time using a Synergy4-BioTek reader. For dose–response experiments drugs were diluted as described for the 96-well assay.

Results

Stable production of bioluminescent P. falciparum gametocytes and expression of the CBG99 luciferase in sexual differentiation

The *pfelo1* locus of the gametocyte producer clone 3D7 was modified by homologous integration of a plasmid carrying the CBG99 luciferase under the control of the flanking genomic regions of the gametocyte-specific gene *pfs16* (Figure 1a). Immunofluorescence and live single-cell bioluminescence experiments showed CBG99 luciferase activity in ~80% of gametocytes of this line, named 3D7*elo1-pfs16*-CBG99.[10]

Cultures of the 3D7*elo1-pfs16*-CBG99 line produced gametocytes routinely and reliably with high efficiency, both in flasks and in microplates. Average gametocytaemia levels ranging from 2% to 4% were routinely obtained from parasites kept for as long as 2 months after thawing from liquid nitrogen, both at Università degli Studi, Milan, and Istituto Superiore di Sanità, Rome.

In synchronized cultures, the activity of CBG99 luciferase starts to be up-regulated at induction of gametocytogenesis and accompanies gametocyte maturation for 10 days from stage I to stage V (Figure 1b). The steady increase in bioluminescent signal due to accumulation of the CBG99 enzyme results in high luciferase activity for the entire gametocyte maturation period, which makes this line suitable for testing compounds against any stage of sexual differentiation.

Due to the potent CBG99 luciferase signal, titration experiments showed that as few as 1000 gametocytes diluted in 10^6 uninfected red blood cells in 10 mL of culture medium can be reliably detected, generating a signal-to-background ratio of at least 3 (Figure 1c). This threshold of sensitivity is lower than that observed with other luciferase reporter assays on asexual or sexual stages from *P. falciparum* luciferase transgenic lines.[8,15]

Use of a non-lysing luciferin substrate improves performance and reliability of the GC-LUC assay

To avoid interference by enhancers and stabilizers in the bioluminescent signal (e.g. luciferase inhibitors, lysing components, ATP)

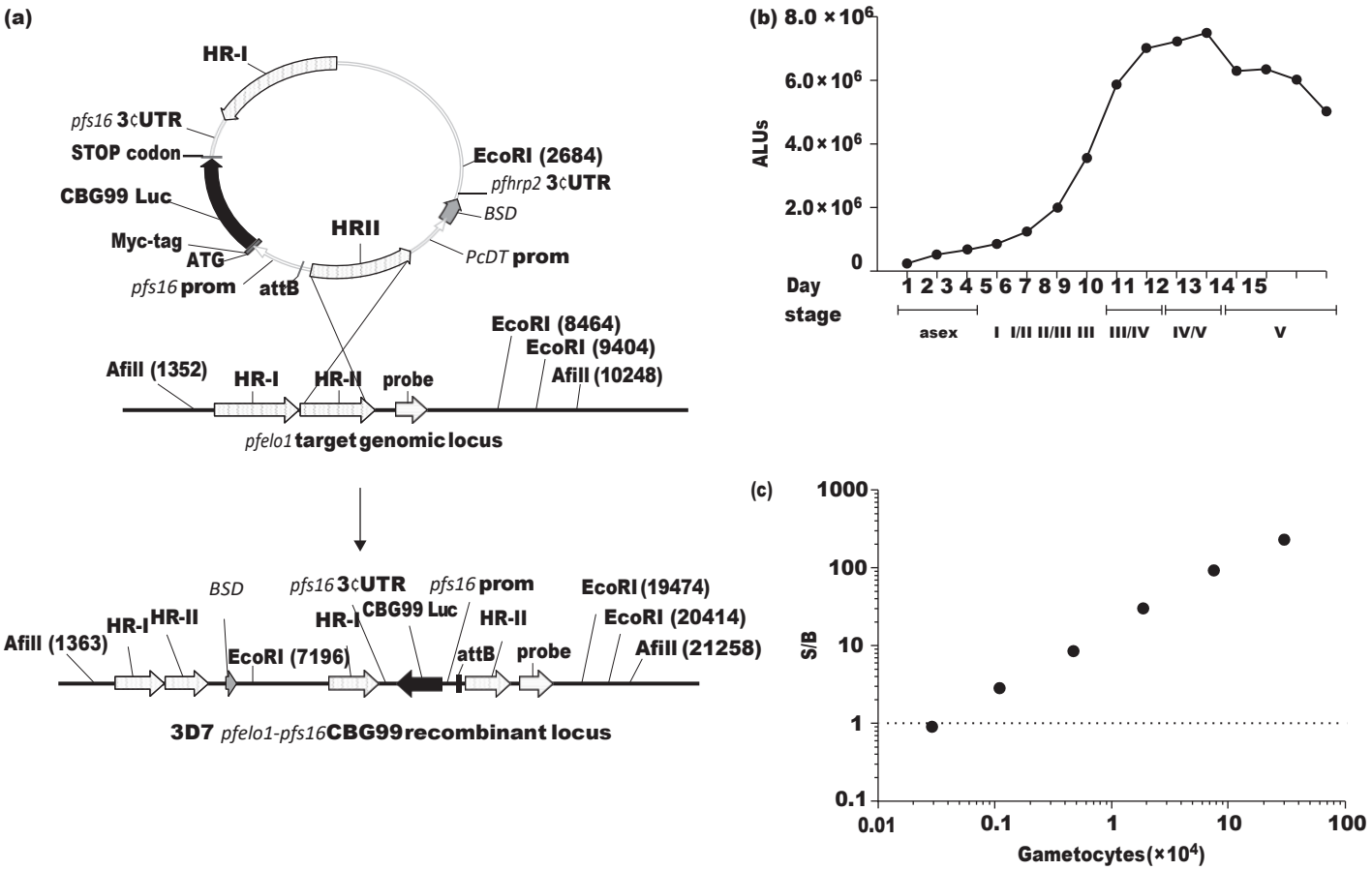


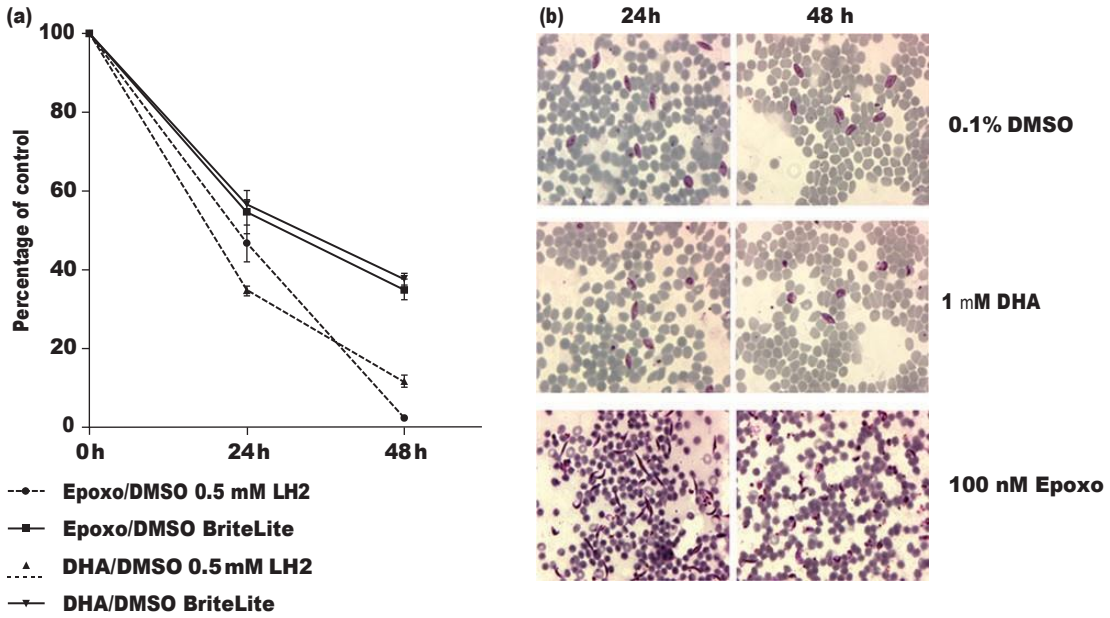
Figure 1. Generation of the *P. falciparum* 3D7*elo1-pfs16*-CBG99 line. (a) Diagram of the *pfs16*-CBG99 integration plasmid and the target *pfelo1* locus before and after homologous recombination-mediated plasmid integration. (b) Time course analysis of bioluminescence in synchronized gametocytes of the 3D7*elo1-pfs16*-CBG99 line. Parasites were grown to high parasitaemia to induce gametocytogenesis. Daily blood smears were obtained to assess asexual and sexual stage parasite composition. At the onset of gametocytogenesis, NAG was added to eliminate residual asexual parasites (days 4 – 5). Two-hundred microlitres of the parasite culture (3% haematocrit, 2% gametocytaemia) was used daily to obtain luciferase measurements with Britelite™ substrate. (c) Titration of the bioluminescent signal in serially diluted 3D7*elo1-pfs16*-CBG99 gametocytes. Percoll-purified stage III gametocytes were counted and serially diluted in microwells using a suspension of red blood cells in incomplete medium. Wells used for the luciferase assay contained 10^6 uninfected erythrocytes in 10 mL and the number of purified gametocytes is indicated on the *x*-axis. Error bars are SDs from triplicate wells. S/B, signal-to-background ratio.

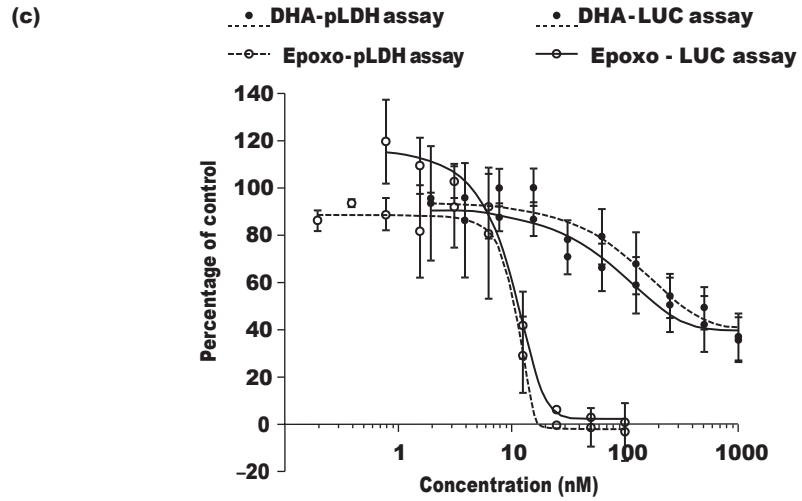
present in luciferase commercial substrates, a novel non-lysing, ATP-free $_D$-luciferin formulation was introduced in the GC-LUC assay. In the absence of cell lysis and exogenous ATP, the bioluminescent signal relies entirely on the ability of the healthy gametocyte to produce both the luciferase and the ATP required for light emission, resulting in a more reliable readout of gametocyte viability.[10] Luciferase activity revealed with the commercial Britelite™ substrate or the $_D$-luciferin formulation was compared after 24 and 48 h of treatment of synchronous 3D7*elo1-pfs16*-CBG99 stage III gametocytes with anti-gametocyte drugs (1 mM dihydroartemisinin or 100 nM epoxomicin). The decline in luciferase activity in the drug-treated gametocytes was significantly more pronounced when using $_D$-luciferin solution compared with Britelite™ (Figure 2a). Importantly, this was observed also at timepoints (e.g. 24 h) at which the morphology of the affected gametocytes was virtually indistinguishable from that of the control parasites (Figure 2b).

The IC_{50} values for dihydroartemisinin and epoxomicin were then compared using the improved luciferase assay and the previously established pLDH assay on late-stage gametocytes. Complete sigmoidal curves obtained with both assays (Figure 2c) yielded very similar IC_{50} values. The highest concentration of dihydroartemisinin (1000 nM), known to kill early-stage gametocytes[4] totally, did not completely kill late-stage gametocytes.[16]

Adaptation of the GC-LUC assay to the 384-well format for HTS

Experiments to scale up the GC-LUC assay to the 384-well format suitable for HTS showed that the minimum usable parasite number was 10000 gametocytes/well (Figure 3a) and that the bioluminescent signal was stable 5 – 30 min after substrate addition. The IC_{50} values of the reference compounds epoxomicin and dihydroartemisinin were comparable to those obtained in 96-well plates (Figures 3b and 2c). Scaling down the assay incubation volumes (100, 50, 40 mL) did not affect accuracy of

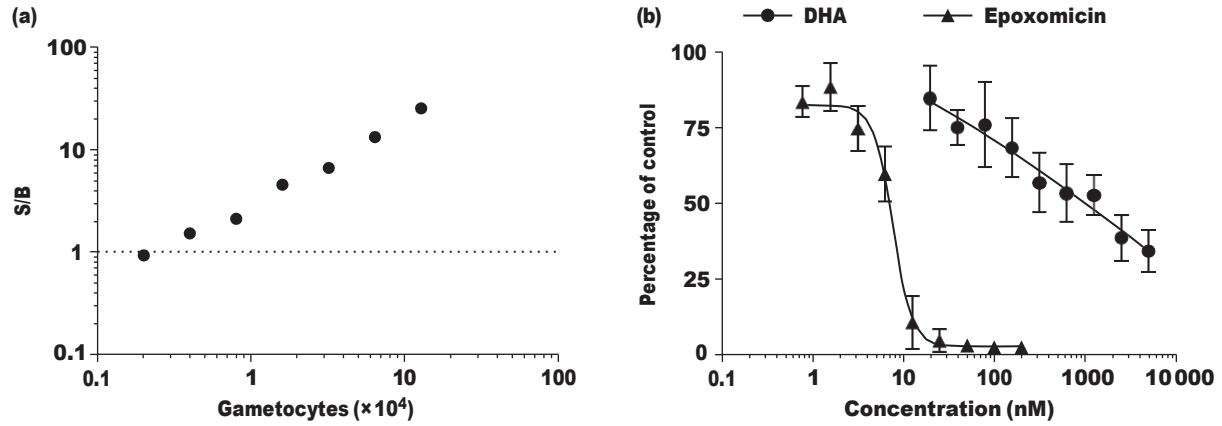




IC_{50}	LUC assay	pLDH assay
DHA	435.3 ± 286.8	490.2 ± 240.0
Epoxomicin	11.6 ± 2.8	7.9 ± 5.0

Figure 2. Establishment of the GC-LUC assay: comparison of luciferase substrates and dose–response effect of the standard anti-gametocyte drugs dihydroartemisinin and epoxomicin on 3D7*elo1-pfs16*-CBG99 gametocytes. (a) Percentage of gametocytes compared with DMSO-treated controls at 0, 24 or 48 h. Circles, 1 mM dihydroartemisinin (DHA); triangles, 100 nM epoxomicin (Epoxo). Solid lines, Britelite™ substrate; dashed lines, non-lysing D-luciferin formulation. Error bars are SDs from three replicate measurements. (b) Representative images of Giemsa-stained smears from treated and untreated gametocytes from the experiment in panel a. (c) Dose– response curves obtained by treating gametocytes with increasing doses of dihydroartemisinin (range 1.9 – 1000 nM, filled circles) or epoxomicin (range 0.78 – 100 nM, open circles) and evaluating parasite viability by the luciferase (continuous line) or pLDH assay (dashed line). Results are expressed as percentages of gametocytes compared with untreated controls. Data are mean+SD from two independent experiments in duplicate or triplicate. The table reports IC_{50} values from the same experiments. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

IC_{50} determination (5.84, 5.91 and 5.89 nM, respectively, for epoxomicin). The level of the bioluminescence signal was negatively affected by increasing the haematocrit, probably due to light absorbance from haemoglobin,[17] with a 30% difference in light units between 2% and 0.5% haematocrit. Assay optimization resulted in the following final conditions: minimum of 10000 gametocytes/well in black plate; haematocrit 2%; final incubation volume 40 mL; sample:D-luciferin ratio 1:1. After drug incubation, the well content was re-suspended, D-luciferin substrate was added and the plate was read after 10 min of incubation in the



	IC_{50}
DHA	622.6 ± 288.1
Epoxomicin	6.6 ± 1.6

Figure 3. Adaptation of GC-LUC assay to 384-well format. (a) Titration of the bioluminescent signal in serially diluted 3D7*elo1-pfs16*-CBG99 gametocytes. Stage V gametocytes were counted, diluted to 2% haematocrit and serially diluted in 384 wells using a suspension of 2% haematocrit red blood cells in complete medium. The number of purified gametocytes is indicated on the *x*-axis. The results are the mean+SD of two independent experiments in quadruplicate. S/B, signal-to-background ratio. (b) Dose– response curves obtained by treating gametocytes with increasing concentrations of dihydroartemisinin (DHA; range 19.5 – 5000 nM, circles) or epoxomicin (range 0.78 – 200 nM, triangles) and evaluating parasite viability by the luciferase assay using the non-lysing D-luciferin substrate. Results are expressed as percentages of gametocytes compared with untreated controls. Data are mean+SD from two independent experiments in triplicate or quadruplicate. The table reports IC_{50} values from the same experiments.

dark. Quality of the HTS adaptation of the GC-LUC assay was confirmed by a Z' factor of 0.70+0.07.

Comparison of the GC-LUC and pLDH assays in measuring anti-gametocyte activity of a reference set of antimalarial drugs

A reference set of 48 drugs (MMV Validation Set) belonging to different chemical classes and showing activity against *P. falciparum* asexual stages was established by MMV for testing on sexual stages.[12] A parallel primary screening (5 mM) of this drug set was performed against early and late gametocyte stages with the 3D7*elo1-pfs16*-CBG99 transgenic line, performing the GC-LUC and the pLDH assay on samples from the same 96-well microplates. The inhibition of gametocyte viability is reported in Figure 4 and Table S1.

The Z' factors calculated in these experiments, 0.87+0.08 for the GC-LUC assay and 0.6+0.1 for the pLDH assay, confirmed the high quality of both assays. In the screening on early-stage gametocytes, 20 drugs in the GC-LUC assay and 22 in the pLDH assay inhibited gametocyte viability by ≥50%, with 19 compounds consistently identified by both assays (Table 1). Of these, 16 showed inhibition of ≥75%, 11 detected only by the GC-LUC assay and 5 by both assays. In the screening on late-stage gametocytes, fewer drugs showed ≥50% inhibition, with 8 and 15 identified by the GC-LUC and pLDH assay, respectively. Among these, eight were shared. The subset of five drugs inhibiting late-stage gametocytes by ≥75% was identified by both assays. Four of these compounds were artemisinin derivatives and the fifth was pentamidine. Only methylene blue (≥95% inhibition in the GC-LUC assay) could not be compared since at this concentration it interferes with the pLDH reading.

The correlation between the values of gametocyte inhibition obtained with the two methods was generally excellent: R^2≥0.85 for early-stage and R^2≥0.90 for late-stage gametocytes (Figure 5a and b). The higher correlation between the assays on late-stage gametocytes is likely to be due to the higher signal-to-background ratio of the GC-LUC assay in late compared with early stages, whereas this was similar between the two stages in the pLDH assay. This difference was due to the observed increase in luciferase expression during sexual development (Figure 1b), whereas pLDH activity was constant during gametocytogenesis (data not shown).

Stage-specific differences in activity of the MMV Validation Set

The comparison of compound activity against early or late gametocyte stages (Figure 5c and d) showed that both assays consistently identified compounds, mostly artemisinin derivatives, that inhibited both stages by ≥50% (right upper quadrant), 9 by the GC-LUC assay and 12 by the pLDH assay (Figure 5c and d). Both assays consistently showed that early-stage gametocytes are generally more susceptible than late-stage gametocytes to the drugs of the MMV Validation Set (right lower quadrants). In the GC-LUC assay 12 compounds, mostly 4-aminoquinolines and amino alcohols, inhibited early- but not late-stage gametocytes by ≥50%, and 10 compounds showed the same inhibition pattern in the pLDH assay (overlap of the two sets was 8 compounds).

Artemisinin derivatives induced complete inhibition of early gametocyte viability by the GC-LUC assay, but not by the pLDH assay. Any direct inhibition of the CBG99 luciferase enzyme by these compounds was ruled out by testing the entire MMV Validation Set on the purified recombinant CBG99 luciferase (Figure S1).

(a)
Luc early GCTs
% inhibition
0
20
40
60
80
100
Artesunate
Artenimol (DHA)
Artemisone
Artemether
OZ277(RBX-11160)
Artemisinin
OZ 439 mesylate
Hydroxychloroquine
Amodiaquine
AQ-13
Chloroquine
Pyronaridine
Pentamidine
Cycloheximide
Piperaquine
Quinine sulfate
Lumefantrine
X?
Mefloquine (Racemic)
Mefloquine (+ RS)
Halofantrine
Naphthoquine
Thiostrepton
NPC-1161B
Pamaquine
Chlorproguanil HCl
Azithromycin
Primaquine
Tafenoquine
Tetracycline
Trans-Mirincamycin
Doxycyclin
Clindamycin
Fosmidomycin
Riboflavin
Sulfadoxine
Deferoxamine
Sulfamethoxazole
N-acetyl-D-penicillamine
Cis-Mirincamycin
Proguanil HCl
Sulfadiazine
Dehydroepiandrosterone
Trimethoprim
Dapsone
P218.HCl
Atovaquone
Pyrimethamine
Amino alcohols
4-aminoquinolines
8-aminoquinolines
Antifolates
Antibiotics
Others
Sulfadoxine
Artemisinins
(b)
pLDH early GCTs
% inhibition
0
20
40
60
80
100
AQ-13
Chloroquine
Artemisone
Artenimol (DHA)
Artesunate
Artemether
Mefloquine (+ RS)
Artemisinin
Quinine sulfate
OZ277(RBX-11160)
Pyronaridine
Pentamidine
Amodiaquine
Mefloquine (Racemic)
Naphthoquine
OZ 439 mesylate
Tafenoquine
Hydroxychloroquine
Piperaquine
Halofantrine
X?
Cycloheximide
Lumefantrine
NPC-1161B
Primaquine
Pamaquine
Chlorproguanil HCl
Trans-Mirincamycin
Proguanil HCl
Azithromycin
Thiostrepton
Atovaquone
Tetracycline
P218.HCl
Pyrimethamine
Cis-Mirincamycin
Sulfadiazine
Fosmidomycin
Riboflavin
N-acetyl-D-penicillamine
Trimethoprim
Sulfadoxine
Doxycyclin
Clindamycin
Deferoxamine
Dapsone
Sulfamethoxazole
Dehydroepiandrosterone
(c)
Luc late GCTs
% inhibition
0
20
40
60
80
100
Artemisone
Artesunate
Artenimol (DHA)
Artemether
Pentamidine
Artemisinin
OZ277(RBX-11160)
OZ 439 mesylate
NPC-1161B
Cycloheximide
Thiostrepton
Pamaquine
Naphthoquine
Pyronaridine
Chloroquine
AQ-13
Amodiaquine
Hydroxychloroquine
Piperaquine
Quinine sulfate
Primaquine
Mefloquine (Racemic)
Mefloquine (+ RS)
Chlorproguanil HCl
Tafenoquine
X?
Lumefantrine
Halofantrine
Azithromycin
Atovaquone
Trans-Mirincamycin
Cis-Mirincamycin
Dapsone
Deferoxamine
Proguanil HCl
Riboflavin
Doxycyclin
Sulfadiazine
Fosmidomycin
Dehydroepiandrosterone
Sulfamethoxazole
Trimethoprim
N-acetyl-D-penicillamine
Clindamycin
Pyrimethamine
Sulfadoxine
P218.HCl
Tetracycline
(d)
pLDH late GCTs
% inhibition
0
20
40
60
80
100
Artesunate
Artemisone
Artenimol (DHA)
Pentamidine
Artemether
Artemisinin
OZ 439 mesylate
OZ277(RBX-11160)
NPC-1161B
Thiostrepton
Quinine sulfate
Naphthoquine
Mefloquine (+ RS)
Cycloheximide
Pamaquine
Pyronaridine
Primaquine
AQ-13
Mefloquine (Racemic)
Piperaquine
X?
Lumefantrine
Hydroxychloroquine
Amodiaquine
Chlorproguanil HCl
Chloroquine
Atovaquone Halofantrine
Tafenoquine
Azithromycin
Dehydroepiandrosterone
Dapsone
Fosmidomycin
Proguanil HCl
Sulfamethoxazole
Riboflavin
Cis-Mirincamycin
Clindamycin
Trans-Mirincamycin
N-acetyl-D-penicillamine
Doxycyclin
Pyrimethamine
Deferoxamine
Sulfadoxine
P218.HCl
Sulfadiazine
Trimethoprim
Tetracycline

Table 1. Number of MMV Validation Set compounds that inhibited early- or late-stage gametocyte viability by 50% or 75% using either the pLDH or the GC-LUC assay

	Inhibition of gametocyte viability			
	≥50%		≥75%	
	individual	shared[a]	individual	shared[a]
Early gametocytes				
GC-LUC[b]	20	19	16	5
pLDH[c]	22		5	
Late gametocytes				
GC-LUC	8	8	5	5
pLDH	15		5	

[a]Number of compounds selected by both assays.
[b]The GC-LUC assay was conducted for 72 h.
[c]The pLDH assay was conducted for 72 + 72 h. Methylene blue was excluded because this compound interfered with the signal of the pLDH assay.

Dose–response experiments using the GC-LUC and pLDH assays for compounds active against late-stage gametocytes

The high correlation between results of the pLDH and GC-LUC assays prompted us to further test the complementarity of the two assays by comparing dose–response curves and IC_{50} values of selected compounds from the MMV Validation Set and the MMV Malaria Box. In view of the importance of screening for new drugs active against the sexual stages directly responsible for mosquito transmission, the assays were performed on late-stage gametocytes. In this analysis, seven of the most active compounds of the MMV Validation Set were selected. Amongst artemisinin derivatives, only dihydroartemisinin, the active metabolite of most artemisinins, artesunate, the most used in clinics, and the new derivative artemisone were selected. In addition, 14 compounds were selected for this analysis from the 400 compounds of the Malaria Box. They were chosen as they were active (≥50% inhibition) in primary screening of the Malaria Box conducted on late-stage gametocytes with the pLDH assay (S. D'Alessandro, S. Parapini, N. Basilico, Y. Corbett and D. Taramelli, unpublished results) and also as they had previously been tested in the SMFA. The IC_{50} values were determined with both assays for the above compounds (Table 2; dose–response curves in Figure S2). The R^2 values of 0.98 and 0.86 obtained for the compounds of the Validation Set and the Malaria Box, respectively, confirmed an excellent correlation between the IC_{50} values determined in the pLDH assay and the GC-LUC assay (Figure S3).

The IC_{50} values of the Malaria Box compounds obtained in both assays were also compared with available SMFA results to evaluate how predictive this was of transmission-blocking activity in mosquitoes. This exercise was extended to all of the 22 Malaria Box compounds for which SMFA data are available (ChEMBL: http://www.ebi.ac.uk/chembldntd and[18,19]) and included a comparison with the IC_{50} values obtained in seven additional gametocyte assays (Table S2).

Out of the 22 compounds, 8 were inactive in the above primary pLDH screening; 4 of these were inactive and 1 was partially active in the SMFA, while the remaining 3 achieved ≥80% inhibition of oocyst intensity (number of oocysts per mosquito). Interestingly, none of the latter three compounds was active in six out of seven additional gametocyte assays, suggesting that these compounds do not impair cell viability, but affect some critical events occurring after gametogenesis.

The comparison between SMFA results and the IC_{50} values for the 14 compounds active in the primary pLDH screening showed the following. Of the 10 compounds that showed, with one exception, MMV000248, a submicromolar IC_{50} in both assays (range 520–940 nM in the LUC assay), 7 showed transmission-blocking activity of ≥80% and one blocked at 50%. In contrast, the remaining two compounds, with IC_{50} values around 900 nM, were inactive in the SMFA. Finally, of the four least active compounds, with IC_{50} values between 3 and 5 mM in both assays, three were reported to be inactive in the SMFA and one had transmission-blocking activity (Table S2).

In conclusion, 8 out of the 10 top ranking compounds with a submicromolar IC_{50} were active as transmission blockers, generally indicating high predictive value for the readout of both the LUC and the pLDH assay. In the comparative analysis of the available IC_{50} values from other published assays, the correlation with SMFA results was smaller (Table S2).

The analysis also showed that compounds with IC_{50} values $\geq$1 mM in both assays failed, with one exception, to block transmission, suggesting that this could be set as a threshold in our assays to confidently predict the transmission-blocking potential of compounds.

Discussion

The need to develop transmission-blocking drugs in the framework of malaria eradication requires the establishment of cell-based assays able to combine a reliable readout, faithfully capturing a compound's ability to kill or inhibit sexual stages, with a cheap, sensitive and robust protocol, truly scalable to HTS level. Here, we examined the correlation between the readouts of two recently developed assays, both meeting the above requirements and adapted to 384-well plates. The first was based on the detection of gametocyte LDH in any *P. falciparum* line or isolate[7] and the second introduced an improved D-luciferin substrate formulation to measure the bioluminescent signal of a novel luciferase reporter specifically expressed in gametocytes.[10]

A variety of assays[4] were designed to measure the ability of compounds to kill the non-dividing sexual stages of *P. falciparum*, particularly the long-lasting, apparently quiescent stage V gametocytes. However, most of these are high-content screening assays[18–22] and require monoclonal antibodies and/or large

Figure 4. Primary screening of the MMV Validation Set (5 mM) on early- and late-stage gametocytes (GCTs). Inhibition of the viability of early (a, b) and late (c, d) gametocytes from the 3D7*elo1-pfs16*-CBG99 transgenic strain measured by luciferase (a, c) or pLDH (b, d) assay. Data are expressed as percentage inhibition compared with untreated controls and represent the mean of three independent experiments performed in duplicate plates. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

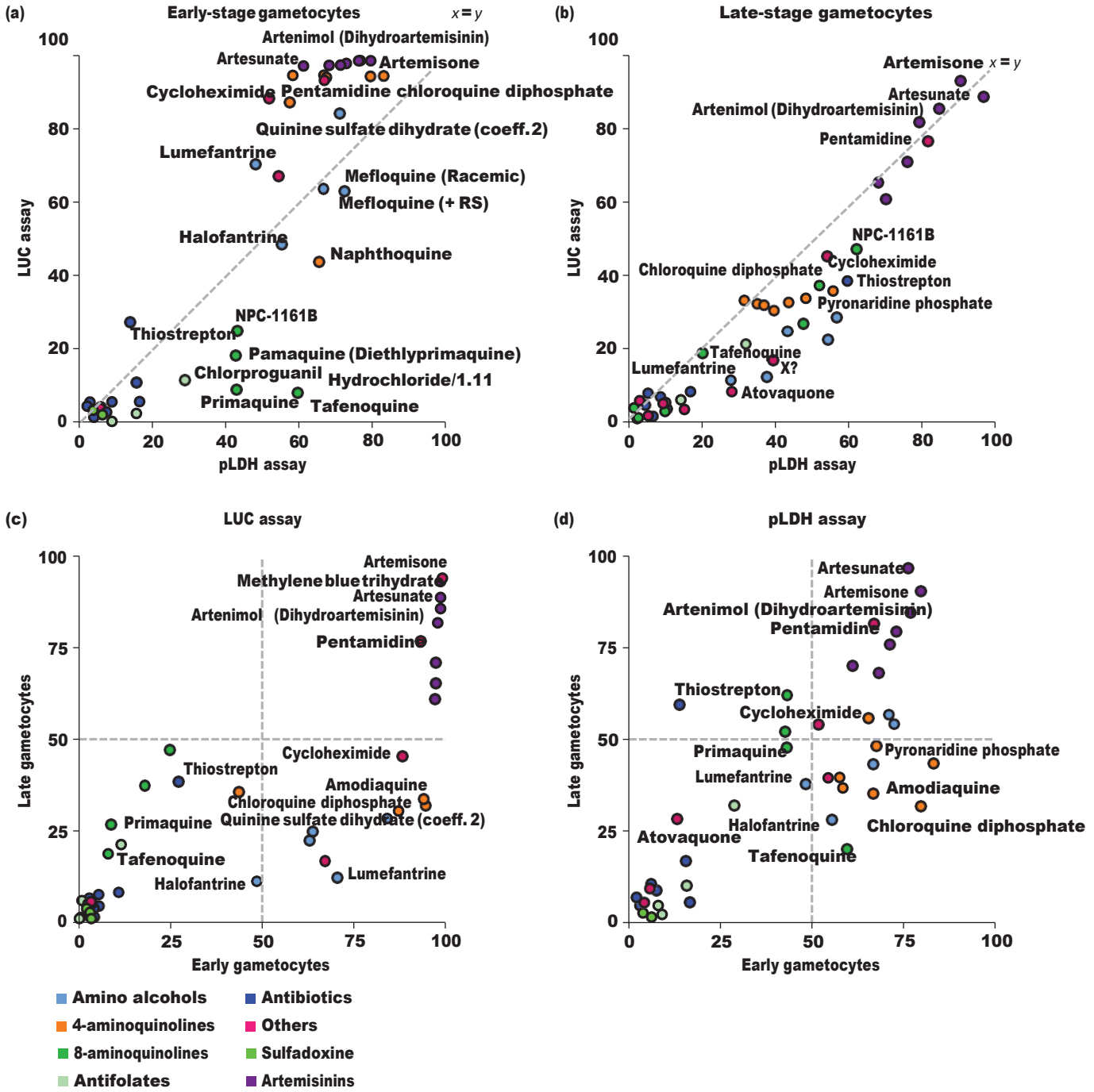


Figure 5. Comparison of primary screening of the MMV Validation Set (5 mM) between luciferase and pLDH assays and between early- and late-stage gametocytes. Inhibition of early (a) and late (b) gametocyte viability measured by the luciferase versus pLDH assay. The results are expressed as percentages of inhibition compared with untreated controls and represent the mean of three independent experiments. The correlation between the two assays is expressed as R^2 (a, R^2 ¼ 0.8541; b, R^2 ¼ 0.90) calculated for the best-fitting line (curve equation in a, y ¼ 1.280x 2 5.024; in b, y ¼ 0.912x 2 5.299). Comparison of the percentage inhibition obtained in the luciferase (c) and pLDH (d) assays in early- versus late-stage gametocytes. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

robotic imaging systems, which limits their suitability for efficient and cost-effective HTS campaigns for large compound libraries. Cell-based assays relying on enzymatic or luciferase reporter reactions are comparatively more amenable to scaling up. In malaria drug discovery, the pLDH assay has been successfully used to screen nearly 2 million compounds against asexual parasites[6] and luciferase reporter lines have been used to develop cell-based assays with asexual and sexual stages.[8–10,23,24] Here we show that

Table 2. *In vitro* activity of selected compounds from the MMV Validation Set and the MMV Malaria Box against *P. falciparum* late-stage gametocytes

	IC_{50} (nM)[a]	
	pLDH assay	LUC assay
MMV Validation Set compounds		
artemisone	17.7+5.2	31.0+4.8
artesunate	36.2+11.5	117.8+13.4
pentamidine	1492.1+149.6	2240.5+121.7
dihydroartemisinin	490.2+240.0	435.3+286.8
NPC-1161B	4451.5+546.5	6860.3+84.3
cycloheximide	5325.3+1160.0	7838.4+1220.6
piperaquine phosphate	16113.0+3481.3	19294.0+879.4
Malaria Box compounds		
MMV000248	2079.8+880.9	605.6+107.1
MMV000448	3136.6+569.7	2427.0+275.1
MMV006172	732.0+269.5	713.8+257.3
MMV007591	3220.6+926.4	3421.0+1004.7
MMV011438	6522.6+2373.2	5115.0+2646.5
MMV019266	2642.0+644.9	3414.0+1171.8
MMV019881	287.7+109.2	752.5+62.3
MMV019918	406.9+70.8	539.7+65.3
MMV085203	235.7+79.4	439.7+104.3
MMV665830	575.7+146.0	811.5+188.7
MMV665882	757.7+278.0	906.5+164.8
MMV665941[b]	157.9+54.7	885.1+352.9
MMV666125	902.2+570.0	942.9+551.5
MMV667491	696.4+269.7	880.8+291.1
Control drug		
epoxomicin	6.69+1.90	12.84+3.71

[a]Data are mean +SD of at least two independent experiments in duplicate.
[b]Methylrosaniline, crystal violet.

both assays demonstrate high quality in medium-throughput screening of anti-gametocyte compounds, with a Z' factor of 0.87+0.08 in 96-well format and 0.70+0.07 in 384-well format for the GC-LUC assay and 0.6+0.1 for the pLDH assay. The lower Z' factor obtained in this set of experiments with the pLDH assay compared with previous work[7] is probably due to modifications of the assay's standard operating procedures. In the GC-LUC assay high sensitivity was confirmed, allowing the use of $\geq$5000 gametocytes per well. Importantly, sensitivity is coupled to the specificity of the bioluminescence readout, ensured by the stage-specific expression of CBG99 luciferase. Several advantages make both assays suitable for HTS. Neither assay requires gametocyte purification prior to the enzymatic or luciferase reaction. In both assays the luciferase and pLDH reaction times are in the order of seconds and minutes, respectively, compatible with a fast and simple HTS protocol. The only drawback is that the pLDH assay requires a post-treatment incubation of 72 h without drugs, adding one medium replacement step to the protocol. Finally, the minimal handling of the small culture volumes, the lack of cell purification and the fairly cheap reagents together make the personnel and material costs of both assays affordable and sustainable also in developing countries. For instance, the introduction of the D-luciferin solution cuts the cost of the luciferase substrate, while the high CBG99 luciferase signal makes it possible to use black microwell plates, which are cheaper than the white ones, used in luminescent measures to enhance the signal.

The main outcomes of this work are that readouts of the pLDH and GC-LUC assays are highly comparable, with correlation coefficients around 0.9 in IC_{50} determinations for a total of 29 drugs and compounds, and that IC_{50} values in the submicromolar range have very good predictivity of the compound's activity in blocking parasite transmission in mosquitoes. The parallel use of these assays on immature and mature gametocytes produced a consistent profile of the susceptibility of *P. falciparum* transmission stages to compounds during their development. For instance, the observation that several compounds and drugs, including artemisinin derivatives, are more active against early- than late-stage gametocytes[8] was confirmed by our work, particularly in the analysis of the MMV Validation Set. The 4-aminoquinolines, targeting haem detoxification in the parasite food vacuole, are not considered to play a role in highly differentiated late-stage gametocytes. This was confirmed here with a clear decline in their inhibitory activity in the transition from early- to late-stage gametocytes. In a less pronounced manner, and with the exception of artesunate and artemisone, the endoperoxides show a similar loss of activity against late-stage gametocytes. Nucleotide synthesis pathways driven by enzymes such as the dihydrofolate reductase are not considered to be active in differentiated gametocytes, where DNA replication is absent. This hypothesis is supported by the low inhibitory activity of pyrimethamine on the viability of both early- and late-stage gametocytes. Atovaquone inhibits cytochrome bc1 in the mitochondrial respiratory chain, a pathway whose functionality plays a major role in proliferating cells.[25] Here, interestingly, atovaquone was shown not to be very active against gametocytes, suggesting low mitochondrial activity. Protein biosynthesis needs to be maintained in differentiated parasites. This was confirmed by the action of cycloheximide on early- and late-stage gametocytes. Strikingly, the initiation of protein synthesis seemed to be less affected by the inhibitor thiostrepton in early- compared with the late-stage gametocytes. In contrast, other antibiotics, such as trimethoprim, mirincamycin, tetracycline and clindamycin, were not shown to affect early- or late-stage gametocytes. Aminoalcohols such as lumefantrine, halofantrine and mefloquine, the latter shown to potentially target the H^+-ATPase of *Mycobacterium tuberculosis*, were more active on early- compared with late-stage gametocytes. This observation supports the hypothesis that early-stage gametocytes, more than the late ones, rely on the energy liberated by the proton transport dependent-hydrolysis of ATP.

In the screening of the 400 Malaria Box compounds, those identified as active against gametocytes by at least three published assays[19 – 21,26,27] were all identified in our work, whilst all of those active in our assays have been similarly classified in at least one, but more often two or more published assays. A general exception is the variable activity of dihydroartemisinin against gametocytes in several assays, including independent determinations in the present work. This might be due to actual differences in the susceptibility of different gametocyte stages to dihydroartemisinin, but possibly also to artefactual differences amongst assays due to different half-lives of dihydroartemisinin in given medium conditions, e.g. the presence of human serum or solvents, or storage temperature.[28]

Although our assays were not specifically designed to reveal possible differential susceptibility of male and female gametocytes to the test compounds, a concept recently proposed based on the outcome of gametocyte sex-specific assays,[19] a few observations can be made. We observed submicromolar IC_{50} values for MMV085203, reported to be active only against male gametocytes, and for MMV019918 and MMV665830, for which 3–8-fold lower IC_{50} values in male versus female gametocytes were calculated[19] (Table S2). This suggests that our assays are sensitive enough to reveal activity of compounds targeting the less represented subpopulation of male gametocytes; alternatively, these results may suggest that our assay conditions reveal effects of these compounds also on female gametocytes.

The increasing knowledge on the biology of *Plasmodium* sexual stages, acquired also by results of recent screening exercises,[9,29] strongly suggests that specific differences exist compared with the biology of pathogenic asexual parasites. This argues for the importance of undertaking large-scale screening campaigns on vast compound libraries irrespective of preliminary knowledge of their activity against asexual parasites. A possible advantage of 'unbiased' identification of novel anti-gametocyte compounds is that they might turn out to be active against other parasite stages, and may even be effective across *Plasmodium* species barriers. The GC-LUC assay validated here, used as a frontline assay, coupled to the complementary pLDH assay for hit confirmation on non-transgenic gametocytes, represents the backbone of a practicable and sustainable pipeline of sensitive, robust and reliable cell-based assays for the identification of a new generation of transmission-blocking compounds.

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Transparency declarations

None to declare.

Supplementary data

Supplementary Methods, Tables S1 and S2 and Figures S1 to S3 are available at *JAC* Online (http://jac.oxfordjournals.org/).

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