










# In vitro Multistage Malaria Transmission Blocking Activity of Selected Malaria Box Compounds

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**Purpose:** Continuous efforts into the discovery and development of new antimalarials are required to face the emerging resistance of the parasite to available treatments. Thus, new effective drugs, ideally able to inhibit the *Plasmodium* life-cycle stages that cause the disease as well as those responsible for its transmission, are needed. Eight compounds from the Medicines for Malaria Venture (MMV) Malaria Box, potentially interfering with the parasite polyamine biosynthesis were selected and assessed in vitro for activity against malaria transmissible stages, namely mature gametocytes and early sporogonic stages.

**Methods:** Compound activity against asexual blood stages of chloroquine-sensitive 3D7 and chloroquine-resistant W2 strains of *Plasmodium falciparum* was tested measuring the parasite lactate dehydrogenase activity. The gametocytocidal effect was determined against the *P. falciparum* 3D7elo1-pfs16-CBG99 strain with a luminescent method. The murine *P. berghei* CTRP.GFP strain was employed to assess compounds activities against early sporogonic stage development in an in vitro assay simulating mosquito midgut conditions.

**Results:** Among the eight tested molecules, MMV000642, MMV000662 and MMV006429, containing a 1,2,3,4-tetrahydroisoquinoline-4-carboxamide chemical skeleton substituted at N-2, C-3 and C-4, displayed multi-stage activity. Activity against asexual blood stages of both strains was confirmed with values of IC<sub>50</sub> (50% inhibitory concentration) in the range of 0.07–0.13 μM. They were also active against mature stage V gametocytes with IC<sub>50</sub> values below 5 μM (range: 3.43–4.42 μM). These molecules exhibited moderate effects on early sporogonic stage development, displaying IC<sub>50</sub> values between 20 and 40 μM.

**Conclusion:** Given the multi-stage, transmission-blocking profiles of MMV000642, MMV000662, MMV006429, and their chemical characteristics, these compounds can be considered worthy for further optimisation toward a TCP5 or TCP6 target product profile proposed by MMV for transmission-blocking antimalarials.

**Keywords:** *Plasmodium*, multi-stage antimalarials, transmission blocking drugs, malaria elimination

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## Introduction

Despite huge efforts to reduce malaria transmission, the parasitic disease caused by *Plasmodium* species continues to be a global health burden. Estimated 217 million cases and 435,000 deaths occurred worldwide in 2017.<sup>1</sup> Most malaria cases and deaths were registered in Sub-Saharan Africa (90%). Whereas substantial control achievements have been obtained over the last decade, no significant progresses in reducing case incidence were made in all World Health Organization (WHO) Regions between 2015 and 2017. Over the same period, the mortality rates also stalled or decreased slightly according to the country.<sup>1</sup>

The success of malaria control is essentially determined by the availability of funding and the socio-economic conditions of endemic countries. It is also well known that the parasite itself, with its complex biology involving a highly anthropophilic mosquito vector, poses major challenges to the development of durable and effective control tools. *P. falciparum*, the most pathogenic and diffused human parasite species, has developed resistance to all the antimalarial drugs. The first chloroquine-resistant *P. falciparum* strains emerged in 1960 in Thailand and diffused in Africa in the late 70ies, following the global malaria eradication program launched by WHO in the 60ies and 70ies.<sup>2</sup> Resistance to sulfadoxine/pyrimethamine (SP) appeared in Thailand in 1967, the same year of its introduction in the country. Similarly, resistance to mefloquine began to appear in Asia in 1985 around the time the drug became widely available.<sup>2</sup> Since the early 2000, WHO advocates artemisinin combination therapy (ACT) as first-line treatment for uncomplicated malaria; however, initial reports of parasite resistance have been published already in 2009 in Asia.<sup>2</sup> ACTs have been integral to the successes of the global malaria control and at present are essential to maintain these achievements. Thus, the recent updates from the WHO Global Malaria Programme monitoring the emergence and the diffusion of multidrug resistance against the artemisinin derivatives (delayed response) and partner drugs in the Greater Mekong Subregion are raising major concerns on the current malaria chemotherapy strategies with ACTs.<sup>3</sup> In Africa, artemisinin resistance has not been reported to date and first-line ACTs remain efficacious for uncomplicated malaria in all malaria-endemic settings.<sup>3</sup>

Efficacious malaria control is based on integrated strategies targeting the parasite in the human host, and mosquitoes responsible for its transmission. Currently, therapeutic tools include: i. the treatment of uncomplicated malaria cases with ACTs after confirmed diagnosis and supplementation with a single dose of primaquine as a gametocytocidal to reduce transmission; ii. intermittent preventive treatment of malaria in pregnancy using SP; iii. chemoprevention with SP + amodiaquine (AQ+SP) in children in highly seasonal transmission areas and in regions where *P. falciparum* strains are still sensitive to both drugs. Vector control is mainly based on the use of long-lasting insecticidal bed nets, which is, however, threatened by the emergence and diffusion of *Anopheles* populations resistant to synthetic pyrethroids.<sup>1</sup>

Thus, drugs not only are important to cure patients, save lives and prevent malaria in individuals but are

essential public health tools to impact on the intensity of malaria transmission, and thus reduce the overall burden of malaria. In line with global frameworks from WHO and the United Nations, Medicine for Malaria Venture (MMV) – a product development partnership in the field of antimalarial drug research and development – puts one of its strategic focus on bringing forward new tools to counteract resistance, reduce transmission, and ultimately to achieve malaria elimination and help eradication.<sup>4</sup> Based on an integrated drug development model, MMV recently proposed to orient R&D efforts on two Target Product Profiles (TPP) and six different Target Candidate Profiles (TCP).<sup>5</sup> Among them, TCP 5 and TCP 6 focus on transmission-blocking drugs and TPP 1 defines drugs (combinations) that are effective against resistant strains, can cure clinical malaria, stop transmission and prevent relapse in a single encounter.<sup>4</sup>

Transmission blocking drugs may target developing and mature gametocytes in the blood circulation of the human host, early sporogonic stages, namely gametes, zygotes and ookinetes developing in the mosquito blood meal or may be harmful to the mosquito vector itself (endectocides). Since the metabolism of immature sexual stages (male and female gametocytes) is largely similar to that of asexual blood stages, they are susceptible to many classes of schizonticides.<sup>6,7</sup> Mature gametocytes, however, are metabolically down-regulated and thus a much more difficult drug target.<sup>8</sup> By contrast, early sporogonic development involves various life-cycle stages and biological processes (gamete formation, fecundation and zygote formation, ookinete maturation) with a wide range of molecular pathways offering potential drug targets.<sup>8</sup> The same holds true for endectocides, which, after being taken up with blood-feeding, potentially may interfere with various biological processes of the mosquito. Thus, ideally and practically more feasibly, a curative, transmission-blocking treatment should contain compounds capable of killing asexual blood stages as well as immature and mature gametocytes. Drugs acting on early sporogonic stages and endectocides need to display a very long half-life given that mature gametocytes can remain in circulation for more than 1 week<sup>9</sup> and the feeding activity of *Anopheles* populations extends even over much longer periods (weeks to months) which vary according to local transmission patterns.

In order to accelerate antimalarial drug discovery and development, MMV has created the Malaria Box, a group of 400 compounds chosen out of 25,000 active on asexual

blood stages. The Malaria Box was made available for free to interested research laboratories.<sup>10</sup>

To contribute to the joint drug development efforts, we considered the results of antiplasmodial screening of Tanzanian medicinal plants reported by Malebo et al.<sup>11</sup> Tetrahydroisoquinolines (THIQs) isolated from *Annickia kummeriae* (Engl. & Diels) Setten & Maas (Annonaceae) were found to be promising and druggable molecules. THIQs isolated from *A. kummeriae* exhibited significant antiplasmodial activity against chloroquine-resistant strain of *P. falciparum* with IC<sub>50</sub> ranging from 0.08 to 0.24 µg/mL in vitro.<sup>11</sup> THIQs inhibit the growth of *P. falciparum* by affecting polyamine metabolism, in particular through the upregulation of the key catabolic enzyme, spermidine/spermine N1-acetyltransferase (SSAT).<sup>12</sup> Polyamine-biosynthetic enzymes are essential for the growth and survival of malaria parasites and exhibit structural features that differ significantly between the parasites and the human host.<sup>13</sup>

The identified THIQs compounds are druggable small molecules, according to Lipinski's rule, with oral bioavailability and drug-likeness indices.<sup>14–17</sup> Literature review indicated that THIQ is one of the privileged chemical scaffolds, commonly found in nature, endowed good antiplasmodial and pharmacokinetic properties.<sup>18</sup> On these bases, to discover and develop novel malaria transmission-blocking drugs, we selected eight THIQs and related compounds from the Malaria Box, with confirmed in vitro activity against asexual blood stages of *P. falciparum* (IC<sub>50</sub> values <150 nM) and potential inhibitors of polyamine biosynthesis as a drug target. All selected compounds display favorable in vitro pharmacokinetics, diverse chemical scaffolds and pharmacophores, good medicinal chemistry and druggability characteristics. Here, we report on the in vitro activity of the eight selected compounds against mature (stage V) gametocytes and early sporogonic stages and discuss further possible compound development.

## Materials and Methods

### MMV Malaria Box Compounds

Eight compounds, five drug-like (MMV000662, MMV006429, MMV019266, MMV665876, MMV006767, MMV019266) and three probe-like (MMV000642, MMV085583, MMV665827), were procured from MCULE (Mcule Inc., Palo Alto, CA 94301, USA; <https://mcule.com/>).

The structures of the molecules are depicted in Figure 1 and other information is given in Table 1.

The drug-like properties are theoretically confirmed applying the rule-of-5-compliance on physicochemical properties, the rule of thumb for compounds likely to show acceptable oral absorption. These are the four physicochemical quantities that have been used to profile molecules with regard to the likelihood of their becoming effective oral drugs. The drug-like properties score is based on rules derived by the filter proposed in H bond donors (nHDon) ≤5, H-bond acceptors (N + O) in ≤10, molecular weight (MW) ≤500 and AlogP ≤5.<sup>19</sup>

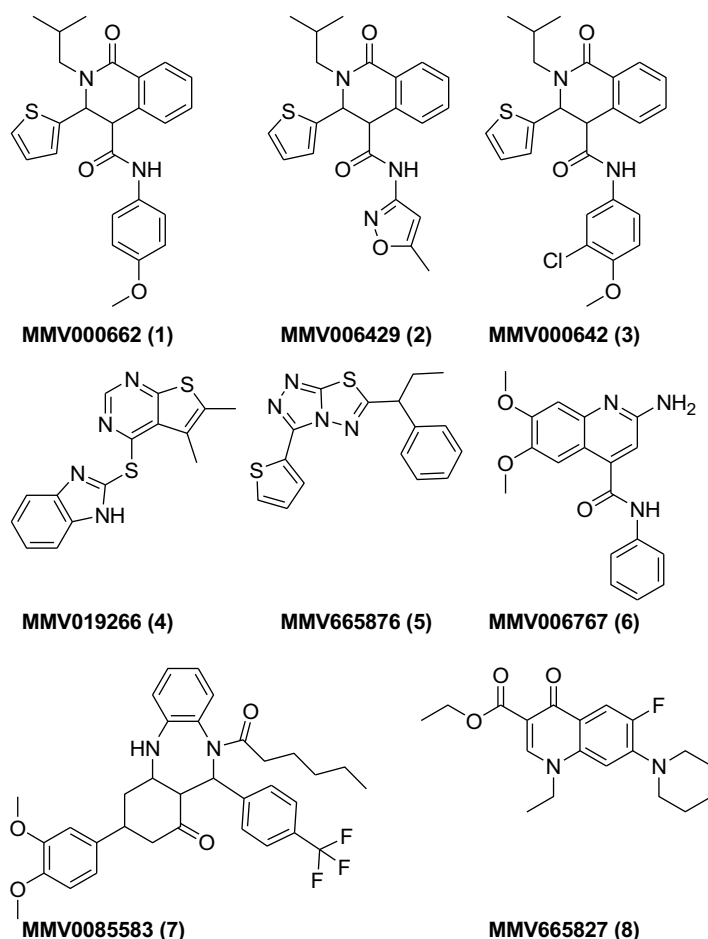
Each of the MMV compounds was profiled with respect to the violation of Lipinski's Rule of 5, N+O (nitrogen count plus oxygen count), molecular weight, the number of hydrogen-bond donors and AlogP. Any compound that failed one or more of these filters was eliminated from the drug-like set and assigned to the probe-like category.<sup>20</sup> Two compounds revealed 1 or 2 violation of the rule of five (Table 2).

### In vitro Antiplasmodial Activity Against *P. falciparum* Asexual Blood Stages Maintenance of *Plasmodium falciparum* Strain W2 and 3D7

Two different strains of *P. falciparum*, W2 (chloroquine-resistant) and 3D7 (chloroquine-sensitive), were used for the chemosensitivity tests. The parasites were cultured according to the method described by Trager and Jensen with slight modifications.<sup>21</sup> The parasites were cultured in human type A-positive or type 0-positive erythrocytes at 5% hematocrit at 37°C in a standard gas mixture consisting of 1% O<sub>2</sub>, 5% CO<sub>2</sub>, 94% N<sub>2</sub>. Medium was made up of RPMI-1640 with the addition of 1% AlbuMax, 0.01% hypoxanthine, 20mM HEPES and 2mM L-glutamine. For routine parasite growth, the parasitemia was maintained within 1% and 5%, and evaluated as the number of infected RBC with respect to the total number counted, in blood smears colored with Giemsa.

### Drug Sensitivity pLDH Assay

For the drug sensitivity assay, the compounds were dissolved in DMSO and then diluted with medium to achieve the required concentrations (final DMSO concentration <1%, which is non-toxic to the parasite). The drugs were placed in 96-wells flat-bottom microplates in duplicate and seven 1:2 serial dilutions made directly in the plate in a volume of 100 µL. Asynchronous cultures with



**Figure 1** Chemical structures of the eight selected Malaria Box compounds taken from Pubmed open chemistry database.

a parasitemia of 1–1.5% and 2% hematocrit (1% final) were aliquoted into the plates and incubated for 72 h, in a final volume of 200  $\mu$ L/well. Chloroquine was used as a reference compound. Parasite growth was determined by measuring the activity of the parasite lactate dehydrogenase (pLDH), according to a modified version of Makler's method.<sup>22</sup> The pLDH activity is distinguishable from the host LDH using the 3-acetyl pyridine adenine dinucleotide (APAD) as co-factor. Briefly, at the end of the incubation, the cultures were carefully re-suspended, and aliquots of 20  $\mu$ L were removed and added to 100  $\mu$ L of the Malstat reagent in a 96-well microplate. The Malstat reagent is made of 0.125% Triton X-100, 130 mM L-lactate, 30 mM Tris buffer and 0.62  $\mu$ M APAD. Twenty-five  $\mu$ L of 1.9  $\mu$ M NBT (Nitro Blue Tetrazolium) and 0.24  $\mu$ M PES (phenazine ethyl sulphate) were added to the Malstat reagent. NBT is reduced to blue formazan and is spectrophotometrically (OD<sub>650</sub>) read as a measure of pLDH activity and thus of parasite viability. The results were expressed as the percent viability compared to the untreated controls, calculated with the following

formula:  $100 \times \frac{([\text{Readout of treated sample} - \text{blank}] / [\text{Readout of untreated sample} - \text{blank}])}{\text{Readout}}$  (Optical Density). As blank, uninfected RBC were used. The percentage of viability was plotted as a function of drug concentrations and the curve fitting was obtained by nonlinear regression analysis using a four-parameter logistic method (software Gen5 1.10 provided with the Synergy4 plate reader [Biotek]). The IC<sub>50</sub>, which is the dose capable of inducing 50% inhibition of parasite viability, was obtained by extrapolation.

## In vitro Activity Against *Plasmodium falciparum* Mature Gametocytes

### *P. falciparum* Gametocyte Induction

The transgenic *P. falciparum* 3D7 strain 3D7elo1-pfs16-CBG99 expressing the *Pyrophorus plagiophthalmus* CBG99 luciferase under a gametocyte-specific promoter was used for chemosensitivity assay on gametocytes. Parasites were cultured and gametocytes obtained as

**Table 1** Information on the Eight Malaria Box Compounds Procured by MCULE

Mcule ID	MMV Code	Classification	Systematic Name	Molecular Weight (MW)	Minimal Purity (%)
MCULE-4901923747	MMV000662	Drug-like	N-(4-methoxyphenyl)-2-(2-methylpropyl)-1-oxo-3-(thiophen-2-yl)-1,2,3,4-tetrahydroisoquinoline-4-carboxamide	434.552	90
MCULE-8458142469	MMV006429	Drug-like	N-(5-methyl-1,2-oxazol-3-yl)-2-(2-methylpropyl)-1-oxo-3-(thiophen-2-yl)-1,2,3,4-tetrahydroisoquinoline-4-carboxamide	409.502	90
MCULE-6374215881	MMV000642	Probe-like	N-(3-chloro-4-methoxyphenyl)-2-(2-methylpropyl)-1-oxo-3-(thiophen-2-yl)-1,2,3,4-tetrahydroisoquinoline-4-carboxamide	468.995	90
MCULE-1940445307	MMV019266	Drug-like	2-({5,6-dimethylthieno[2,3-d]pyrimidin-4-yl}sulfanyl)-1H-1,3-benzodiazole	312.415	90
MCULE-6989044901	MMV665876	Drug-like	6-(1-phenylpropyl)-3-(thiophen-2-yl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole	326.444	90
MCULE-4456901740	MMV006767	Drug-like	2-amino-6,7-dimethoxy-N-phenylquinoline-3-carboxamide	323.345	90
MCULE-5171532492	MMV085583	Probe-like	(3R,11R)-3-(3,4-Dimethoxyphenyl)-10-hexanoyl-11-[4-(trifluoromethyl)phenyl]-2,3,4,5,10,11-hexahydro-1H-dibenzo[b,e][1,4]diazepin-1-one	592.646	90
MCULE-1785799097	MMV665827	Probe-like	ethyl 1-ethyl-6-fluoro-4-oxo-7-(piperidin-1-yl)-1,4-dihydroquinoline-3-carboxylate	346.395	90

**Note:** Malaria Box Compounds as described in Van Voorhis et al.<sup>10</sup>

**Abbreviation:** MMV, Medicines for Malaria Venture.

**Table 2** Drug Like Properties of the Eight Malaria Box Compounds

Malaria Box Compound	Ro5_Violation Count	N Plus O_Count	Molecular Weight	Num_H_Donors	ALogP
1. MMV000662	0	5	434.55054	1	4.427
2. MMV006429	0	6	409.50132	1	3.582
3. MMV000642	1	5	468.9956	1	5.092
4. MMV019266	0	4	312.41258	1	4.458
5. MMV665876	0	4	326.43915	0	4.285
6. MMV006767	0	6	323.34587	2	2.56
7. MMV085583	2	6	592.6479	1	7.336
8. MMV665827	0	5	346.39592	0	3.364

**Notes:** Ro5\_Violation Count = Violation of Lipinski's Rule of 5; eg 1=1 violation of Lipinski's rule; N plus O Count = Sum of Nitrogen and Oxygen atoms; Num\_H\_Donors = Sum of hydrogen-bond donors in the molecule; ALogP = Calculated partition coefficient.

previously described.<sup>23</sup> Late-stage gametocytes were exposed to compounds at day 11 after *N*-acetylglucosamine (NAG) addition. Gametocytes stages were counted in Giemsa stained smears and the percentages of stage V gametocytes were higher than 80%.

## Gametocyte Drug Susceptibility Assay

Compounds were prepared by serial dilution, in 96-well plate, in the complete medium as described for asexual parasites. Methylene blue and dihydroartemisinin were used as reference drugs. After 72 h incubation, luciferase activity

was taken as a measure of gametocytes viability, as previously described.<sup>24</sup> Briefly, drug-treated gametocytes at 2% haematocrit 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 using a Synergy 4 (Biotek) microplate reader. The IC<sub>50</sub> was calculated as described for asexual parasites, being the readout for gametocytes ALU (Arbitrary Luminescent Units). As a blank, gametocytes treated with a high dose of MB (300 ng/mL) were used.

## Activity Against Early Sporogonic Development in vitro on *Plasmodium berghei* Pb.CTRP<sub>p</sub>.GFP Strain *Plasmodium* Strain

The *P. berghei* CTRP<sub>p</sub>.GFP strain, expressing GFP exclusively at early sporogonic stages (ESS), namely in zygotes and ookinetes, was used for the assessment of molecules' activity against the development of ESS in vitro. The rodent malaria parasite strain was maintained by mouse to mouse acyclic and mouse to mosquito to mouse cyclic passages. BALB/c mice and *Anopheles stephensi* mosquitoes were employed as vertebrate and vector host, respectively.

### Vertebrate Host for *P. berghei* CTRP<sub>p</sub>.GFP Gametocyte Production

Eight- to ten-week-old BALB/c mice were used as gametocyte donors for the in vitro experiments. The mice were reared in the animal house (24°C, 14 h light/10 h dark cycle and 70% relative humidity), fed on standard mice pellets (Mucedola s.r.l., Milano, Italy) and provided with tap water *ad-libitum*. Experimental animal rearing and handling were in compliance with the Italian Legislative Decree on the "use and protection of laboratory animals" (D. Lgs. 116 of 10/27/92) and in full adherence with the European Directive (86/609) of 24/11/1986 (license no. 125/94A, issued by the Italian Ministry of Health). The protocol was approved by the University Research Ethics Committee of the University of Camerino – protection of animals used for experimental and other scientific purposes (protocol number: UREC\_CAM\_2017/19). The experiments were in accordance with the protocols approved by the Animal Ethics committee of the University of Camerino.

### Evaluation of Effects on Early Sporogonic Stages in vitro

The impact of MMV molecules on the development of ESS was evaluated in the ookinete development assay.<sup>25</sup> Parasite infected BALB/c mice were used as a source of gametocytes for the in vitro assay and obtained by the following procedure. To stimulate erythropoiesis, mice were treated with phenylhydrazine (120 mg/kg i.p.) 4 days prior to infection with *P. berghei* CTRP<sub>p</sub>.GFP through i.p. injection of 10<sup>7</sup> infected RBCs. Gametocytemia was checked 4-days post-infection by microscopic examination of thin blood films and the maturity of microgametocytes verified by testing their capacity to generate flagellate microgametes in the exflagellation assay. In brief, a drop of tail blood from a gametocytemic mouse was

diluted at a ratio of about 1:25 in exflagellation medium (RPMI 1640 containing 25 mM HEPES, 25 mM sodium bicarbonate, 50 mg/L hypoxanthine, 100 μM xanthurenic acid, pH 8.3). Then, 8 μL of the diluted blood sample was incubated and examined in a hand-made coverslip/slide chamber consisting of a slide as a base, 2 cover slips placed on it laterally as spacers, and a third one placed on the top to close the chamber. Then, all sides of the cover slip were sealed with a mixture of Vaseline and Tween 80 (approximately 1:2 ratios). After 20 min incubation at 19° C, the slides were examined for exflagellation centers under the microscope (400X magnification). Mice with abundant exflagellation centers (more than 3 per 1000 red blood cells) were selected and used as blood donors for the ookinete development assay. This assay allows to assess effects of molecules on the sexual parasite stages developing in the vector, namely male and female gametes, zygotes and ookinetes, simulating in vitro the physicochemical conditions of the mosquito midgut environment: 80 μL of ookinete medium [exflagellation medium with 20% heat-inactivated foetal bovine serum and 1% penicillin (10,000 U/mL)/streptomycin (10,000 μg/mL) adjusted to pH 7.4] was added to the wells of a 96-well microplate (Nunc, Denmark). Dimethyl Sulfoxide (DMSO) was used as a solvent for molecules. Ten microliter of diluted molecules was then added to the microplate wells containing 80 μL of medium to obtain the desired test concentrations (12.5–100 μM). DMSO at 0.2% was used as a negative control. Then, 10 μL aliquots of blood obtained by cardiac puncture from gametocytemic mice were transferred to the microplate wells containing test products or solvent controls, and mixed swiftly. The plates were then incubated at 19°C for 24 h. At the end of incubation, well contents were mixed and 5 μL cell suspension from each well was withdrawn and diluted with PBS (pH=7.4) at about a ratio of 1:25 to 1:50 in a separate 96-well microplate. This dilution step allowed to obtain – after cell settlement – a monolayer of blood cells and parasites, a condition that was required for an accurate microscopic examination. GFP expressing zygotes and ookinetes were visualized using a Zeiss fluorescent microscope (400 X magnification) and quantified with the help of an ocular grid. Each molecule was examined in three replicate wells and six wells were reserved for DMSO controls. Experiments were repeated at least twice with different donor mice. The percent inhibition of ESS development induced by the molecules was calculated as follows:

$$\text{Percent inhibition of ESS development} = 1 - \left( \frac{\text{Mean ESS count in test wells}}{\text{Mean ESS count in solvent control wells}} \right) \times 100$$

## Thin Blood Smear Preparation

In order to assess the morphological impact of active compounds on zygotes and ookinetes, thin blood smears were prepared from the plate wells at the end of the ookinete assay. The MMV molecule treated wells were compared with DMSO controls. Briefly, the content of triplicate wells was transferred into an eppendorf tube and centrifuged at 1500 rpm for 10 min. Thin blood smears were prepared with the cell pellet, fixed with methanol and stained with giemsa (10% in PBS pH 7.4) for 90 mins. Smears were examined under the light microscope (1000×magnification) and early sporogonic stages counted over a slide area corresponding to 80,000 red blood cells. Early sporogonic forms on treatment and control slides were differentially counted as zygotes, retort forms, fully matured ookinetes, denatured zygotes and residual cells.

## Statistical Analyses

Excel 2007 spreadsheet and GraphPad Prism 6 statistical software (GraphPad Software, S. Diego, CA, USA) were used for data analysis. Descriptive statistical tools were also applied to express Early Sporogonic Stage counts and % inhibition values as arithmetic means  $\pm$  95% confidence intervals (95% CI).

## Results

### In vitro Activities of MMV Malaria Box Compounds Against Asexual Stages of *P. falciparum*

One of the selection criteria for the eight studied compounds was high in vitro activity against asexual blood stages ( $IC_{50}$  values below 0.150  $\mu$ M), with the exception of MMV006767 (6), that was included for its structural characteristics although displaying only moderate activity in previously published screening (Table 3). Compound 6 displayed moderate activity ( $IC_{50}$  > 2148  $\mu$ M) also in this study, whereas compounds 1, 2, 3, 7 and 8 confirmed  $IC_{50}$  values in the range of 0.100–0.300  $\mu$ M against both CQ-S and CQ-R *P. falciparum* strains remaining within the in-between laboratory variability (Table 3). Compound 4 (MMV019266) was not active in our assay ( $IC_{50}$  > 8  $\mu$ M) in contrast to the data obtained by the majority of other groups (Table 3 and Van Voorhis et al 2016).<sup>10</sup> The activity of four compounds (1-2-3-7) was better than CQ on the CQ-resistant strains. None of the tested compounds revealed a level of activity comparable to that of

**Table 3** In vitro Activity of Investigated Malaria Box Compounds Against Asexual Blood Stages of W2, CQ-R, and 3D7, CQ-S *P. falciparum* Strains and Comparison with Data in the Literature

Compound Name	<i>P. falciparum</i> Asexual Blood Stages $IC_{50}$ ( $\mu$ M)													
	CQ-Resistant Strains					CQ-Sensitive Strains								
	W2 <sup>a</sup>	Dd2 <sup>b</sup>	W2 <sup>b</sup>	KI <sup>b</sup>	KI <sup>c</sup>	Dd2 <sup>d</sup>	FCR3 <sup>e</sup>	3D7 <sup>a</sup>	3D7 <sup>b</sup>	HB3 <sup>b</sup>	3D7 <sup>f</sup>	3D7 <sup>f</sup>	3D7 <sup>g</sup>	
1. MMV000662	0.133 $\pm$ 0.069	0.053	0.049	0.066	0.074	1.258		0.120 $\pm$ 0.050	0.049	0.058	0.262	0.37	0.343	0.037
2. MMV006429	0.132 $\pm$ 0.038	0.073	0.077	0.079	0.039	0.16	0.074	0.123 $\pm$ 0.003		0.062	0.213	0.385	0.3	0.033
3. MMV000642	0.070 $\pm$ 0.002				0.051	0.046		0.117 $\pm$ 0.012						0.02
4. MMV019266	> 8	0.079	0.062	0.153	0.582	0.844	0.044	>8	0.058	0.093				0.62
5. MMV665876	0.690 $\pm$ 0.048	0.078	0.06	0.123	0.916	0.338	0.158	0.340 $\pm$ 0.016		0.129	0.15	0.181	0.415	0.26
6. MMV006767	2.148 $\pm$ 0.031	0.357	0.797	1.494	0.884	0.884	0.931	1.020 $\pm$ 0.319	0.592	0.812	0.129	0.144		0.93
7. MMV085583	0.101 $\pm$ 0.036	0.245	0.147	0.082	0.288	0.179	0.179	0.171 $\pm$ 0.045	0.165	0.200	0.129			0.24
8. MMV665827	0.295 $\pm$ 0.018			0.063	0.259	0.276		0.173 $\pm$ 0.005	0.165	0.130				0.12
DHA	0.002 $\pm$ 0.001							0.002 $\pm$ 0.001						
CQ	0.304 $\pm$ 0.138							0.022 $\pm$ 0.006						

Notes: <sup>a</sup>Original data from this work. Mean  $\pm$  SD of at least two experiments. <sup>b-c</sup>Data were generated in the laboratories of Ayong, Avery, Horrocks, Van Voorhis and Poulsen and published as original data in the review of Van Voorhis et al. Creative Commons CCO public domain dedication. <sup>10</sup>

Abbreviations: CQ, chloroquine; DHA, dihydroartemisinin.

dihydroartemisinin (DHA), used as a positive control in the assay.

## In vitro Activity of MMV Malaria Box Compounds Against Mature Stage V Gametocytes

Among the eight screened MMV molecules, compounds 1, 2 and 3 (MMV000662, MMV006429, MMV000642), showed  $IC_{50}$  values below 5  $\mu$ M, ranging from 3.43 to 4.42, against stage V gametocytes (Table 4). Compound 6 (MMV006767) displayed  $IC_{50}$  values below 10  $\mu$ M and compounds 4, 5, 7 and 8 (MMV19266, MMV665876, MMV085583, MMV665827) were not active ( $IC_{50} > 20\mu$ M). As shown in Table 4, these results are in good agreement with other studies that examined the impact on stage V gametocytes<sup>26–28</sup> and to a lesser extent with those that employed tests not discriminating gametocyte stage IV and V.

## Effects of MMV Malaria Box Compounds on Early Sporogonic Stages

At a primary screening dosage of 50  $\mu$ g/mL, inhibitory effects on early sporogonic development were observed with compounds 1, 2 and 3 (MMV000662, MMV006429, MMV000642), the same compounds found to be active on asexual stages and gametocytes. At this high dosage (corresponding to the molar concentration of 115.06  $\mu$ M, 122.10  $\mu$ M, 106.61  $\mu$ M for compounds 1, 2 and 3, respectively), the number of early sporogonic stages (ESS), ie the total counts of fluorescent zygotes, retort forms (early ookinete stage) and elongated ookinetes, was reduced by 60% to 80% in wells incubated with the three compounds (Figure 2). In addition, differential counts of retort forms and elongated ookinetes revealed inhibition of ookinete development (>80%) by compounds 1, 2 and 3. Compounds 4 (MMV19266) and 7 (MMV085583) were not active, whereas compounds 5 (MMV665876), 6 (MMV006767) and 8 (MMV665827) appeared to interfere with the ookinete development (>60%), but not with the early sporogonic development before zygote formation as reflected by the overall counts of ESS (<20% inhibition).  $IC_{50}$  values estimated for compounds 1, 2 and 3 were in the range of 30 to 40  $\mu$ M indicating an impact of the three compounds on ESS counts and confirming the activity of these compounds previously reported (Table 5).<sup>29</sup> Just a slightly stronger effect of the three compounds was observed on ookinete development, illustrated by  $IC_{50}$  values ranging from 19 to 29  $\mu$ M (Table 5).

## Effects of Malaria Box Compounds on Early Sporogonic Stages' Morphology

To assess whether the effects of Malaria Box compounds on early sporogonic stages, evidenced in the ookinete development assay (ODA), was accompanied by morphological alterations of the cells, Giemsa stained thin smears were prepared from the ODA plates after treatment with the compounds at 50  $\mu$ g/mL.

On the slides prepared from compounds 1, 2 and 3 (MMV000662, MMV006429 and MMV000642), not a single fully elongated ookinete could be found screening 120 optical fields at 1000x magnification (corresponding to an area covered by a monolayer of 12,000–14,000 red blood cells) (Table 6). For comparison, on the DMSO control smear, 46 mature ookinetes and 30 retort forms were observed among a total of 122 early sporogonic forms. On the slides of compounds 1, 2 and 3, the few ESS retrieved, showed various morphological alterations such as irregular shape, jagged borders, enlarged hemozoin vacuoles and absence of a condensed nucleus (Figure 3, images G and H). On the slides of the cells treated with compounds 5 and 6 (MMV665876, MMV006767), having shown marginal activity against ESS and slight effects on ookinete formation in the ODA, 34 and 33 early sporogonic forms, respectively, were recorded, corresponding to about one fourth of the control (122).

Notably, most of the observed cells were altered zygotes or post-zygotic forms displaying alterations, as illustrated above for compounds 1, 2 and 3 (Figure 3, images E to H). The smear of compound 8 showed 91 ESS, but almost all of them displayed morphological alterations of the zygotes or post-zygotic forms. In contrast to the alterations observed on slides of compounds 1, 2, 3, 5 and 6, early sporogonic forms incubated with compound 8 displayed a centrally condensed nucleus (Figure 3, images I to L).

## Discussion

The purpose of this study was to evaluate the in vitro efficacy of eight selected Malaria Box compounds, potential inhibitors of polyamine biosynthesis, against different stages of the *Plasmodium* parasite. The results from the various stage-specific tests confirmed the activity of some of these compounds against *P. falciparum* asexual blood stages and gametocytes and against *P. berghei* early sporogonic stages. Three out of the eight screened Malaria Box compounds, namely compound 1 (MMV000662),

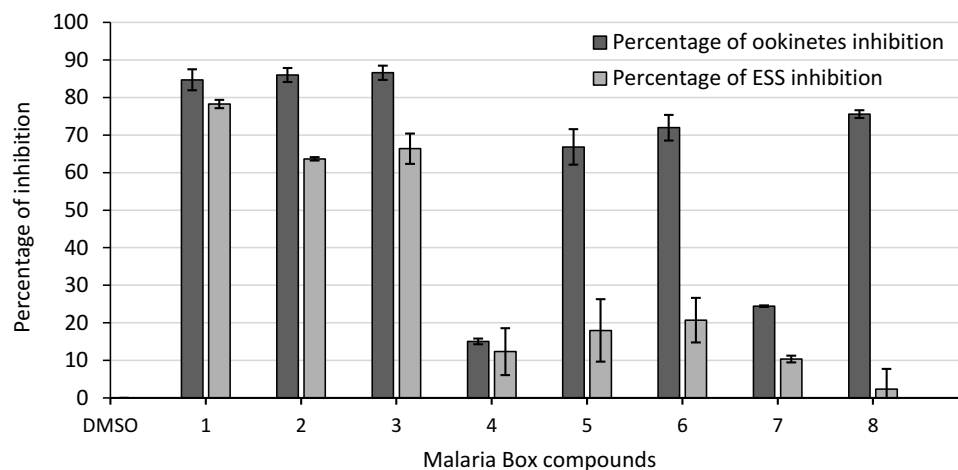


Table 4 In vitro *P. falciparum* Gametocytocidal Activity of MMV Malaria Box Compounds and Comparison with Data in the Literature

MMV compounds	P. falciparum gametocytes					Stage V % Inhibition at 12.5µM <sup>e</sup>	3D7A Female Gamete Formation Acridine Orange % Inhibition at 5µM <sup>f</sup>	Female Gametocyte Stage V % Inhibition of Gamete Formation at 1µM <sup>g</sup>	Male Gametocyte Stage V % Inhibition of Gamete Formation at 1µM <sup>g</sup>
	Stage V 3D7elolipfs16-CBG99 Luciferase Assay IC <sub>50</sub> (µM), 72h <sup>a</sup>	Stage IV-V NF54 Resazurin Assay % Inhibition at 5µM <sup>b</sup>	Stage IV-V 3D7 pLDH Assay % Inhibition at 3.7 µM After 72+72h <sup>c</sup>	Stage IV-V NF54-pfs16-GFP pLDH Assay <sup>d</sup>					
				% Inhibition at	IC <sub>50</sub> nM				
			5µM	0.5µM					
1. MMV000662	3.57±0.61	0	28	53	26	94	69	14	22
2. MMV006429	4.42±1.01	46	22	36	31	90	87	40	52
3. MMV000642	3.43±0.50	0	14	66	55	88	44	-2	9
4. MMV019266	57.60±12.05	35	79	94	31	93	40	-11	31
5. MMV665876	29.72±0.21	56	4	12	9	27	1	-48	1
6. MMV006767	6.03±2.16	0	2	0	3	23	-3	-10	22
7. MMV085583	> 33.75	70	33	61	33	56	8	-11	59
8. MMV665827	> 57.74	52	28	93	91	20	-2	-15	92
DHA	> 10.00								
MB	0.08±0.02								

**Notes:** <sup>a</sup>Original data from this work. Mean ± SD of at least two experiments. <sup>b</sup>Data were generated in the laboratories of Mancama and Taramelli, respectively, and published as original data in the review of Van Voorhis et al; Creative Commons CCO public domain dedication. <sup>c</sup>Data from Duffy et al<sup>30</sup> and reviewed by Van Voorhis et al<sup>10</sup> and reviewed by Van Voorhis et al.<sup>28</sup> <sup>d</sup>Data from Swann et al<sup>27</sup> and Plouffe et al.<sup>28</sup> <sup>e</sup>Data from Lucantoni et al<sup>26</sup> and reviewed by Van Voorhis et al.<sup>10</sup> <sup>f</sup>Data from Ruescher et al<sup>29</sup> and reviewed by Van Voorhis et al.<sup>10</sup> <sup>g</sup>Data from Swann et al<sup>27</sup> and Plouffe et al.<sup>28</sup> and reviewed by Van Voorhis et al.<sup>10</sup>

**Abbreviations:** DHA, Dihydroartemisinin; MB, methylene blue.



**Figure 2** Effects of the eight Malaria Box compounds (1 MMV000662, 2 MMV006429, 3 MMV000642, 4 MMV019266, 5 MMV665876, 6 MMV006767, 7 MMV085583, 8 MMV665827) on early sporogonic development of *P. berghei* CTR<sub>p</sub>-GFP at a screening concentration of 50 µg/mL. Grey bars: impact (% inhibition in respect to DMSO controls) on the formation of early sporogonic stages, counting all forms (zygotes, retort forms and fully elongated ookinetes); black bars: impact (% inhibition) on the formation of ookinetes counting elongated forms (retorts, stumpy and fully elongated ookinetes). Vertical bars depict confidence intervals at 95%.

compound 2 (MMV006429) and compound 3 (MMV000642), exhibited inhibitory effects against all the three tested parasite stages, responsible for the disease and for its transmission to the mosquito vector.

Four of these compounds (1, 2, 3 and 7) were active on chloroquine-resistant and chloroquine-sensitive asexual blood stages of *P. falciparum*, with IC<sub>50</sub> values ranging from 0.070 to 0.171 µM. Comparing the present results with those of other studies presented in the Van Voorhis meta-analysis of the total 400 Malaria Box compounds, a high agreement emerges.<sup>10</sup> In particular, looking at 12 studies that examined a majority of the eight herein considered compounds (please see Supporting Information S1 Table. Malaria Box HeatMap available online [https://journals.plos.org/plospathogens/article/file?type=supplemental\\_y&id=info:doi/10.1371/journal.ppat.1005763.s002](https://journals.plos.org/plospathogens/article/file?type=supplemental_y&id=info:doi/10.1371/journal.ppat.1005763.s002); columns

from N to Q and from S to Z in Van Voorhis et al),<sup>10</sup> a full concordance appears in the case of compound 2 and 3, with 10/10 and 5/5 studies, respectively; an almost complete match emerges also for compound 1, active in 9/10 studies and still a good match was noted for compound 7 active in 7/11 different reports.

Compounds 1, 2 and 3 resulted also to be the most active against *P. falciparum* stage V gametocytes with IC<sub>50</sub> values ranging from 3.3 to 4.4 µM. Comparison with studies presented in the Van Voorhis review reveals a clear concordance with tests specifically targeted on mature stage V gametocytes, but a poor match with protocols that include both stage IV and V gametocytes (please see Supporting Information S1 Table. Malaria Box HeatMap columns from AU to CB in Van Voorhis et al).<sup>10</sup> Testing compounds 1, 2, and 3 at 12.5 µM on stage V gametocytes, an inhibition in the range of 88 – 94% was observed by Winzeler and colleagues (S1 Table. Malaria Box HeatMap,<sup>10</sup> column BC)<sup>27</sup> whereas inhibition of gamete formation by stage V gametocytes from 40% to 78% was reported by Alano, Avery and colleagues (S1 Table. Malaria Box HeatMap,<sup>10</sup> column BV)<sup>26</sup> at 5 µM for the same compounds. Moreover, compound 2 at 1 µM was found to inhibit female and male gamete formation upon exposure of stage V gametocytes.<sup>29</sup>

Regarding gametocyte stage IV and V inclusive tests, according to the heat map analysis<sup>10</sup> compound 3 was active only in the Duffy & Avery study displaying a 66% inhibition at 5 µM on stage IV–V gametocytes of the NF54-pfs16GFP strain (S1 Table. Malaria Box

**Table 5** IC<sub>50</sub> Values of Malaria Box Compounds 1 MMV000662, 2 MMV006429 and 3 MMV000642 on Early Sporogonic Stages and Ookinete Development and Comparison with Data in the Literature

Compound Name	IC <sub>50</sub> (µM) [95% C.I.]		<i>P. berghei</i> Ookinete Assay % Inhibition at 10µM <sup>b</sup>
	ESS Inhibition <sup>a</sup>	OD Inhibition <sup>a</sup>	
1. MMV000662	30.8 [28.5–33.8]	19.0 [15–24.7]	100
2. MMV006429	39.8 [35.5–44.2]	26.4 [25.7–27.3]	94,1
3. MMV000642	34.1 [31.8–36.4]	29.1 [0.4–35.7]	80,8

**Notes:** <sup>a</sup>Original data from this work. <sup>b</sup>Data from Rueker et al.<sup>29</sup>

**Abbreviations:** ESS, Early sporogonic stages; OD, Ookinete development.

**Table 6** Effects of Malaria Box Compounds on Early Sporogonic Stages

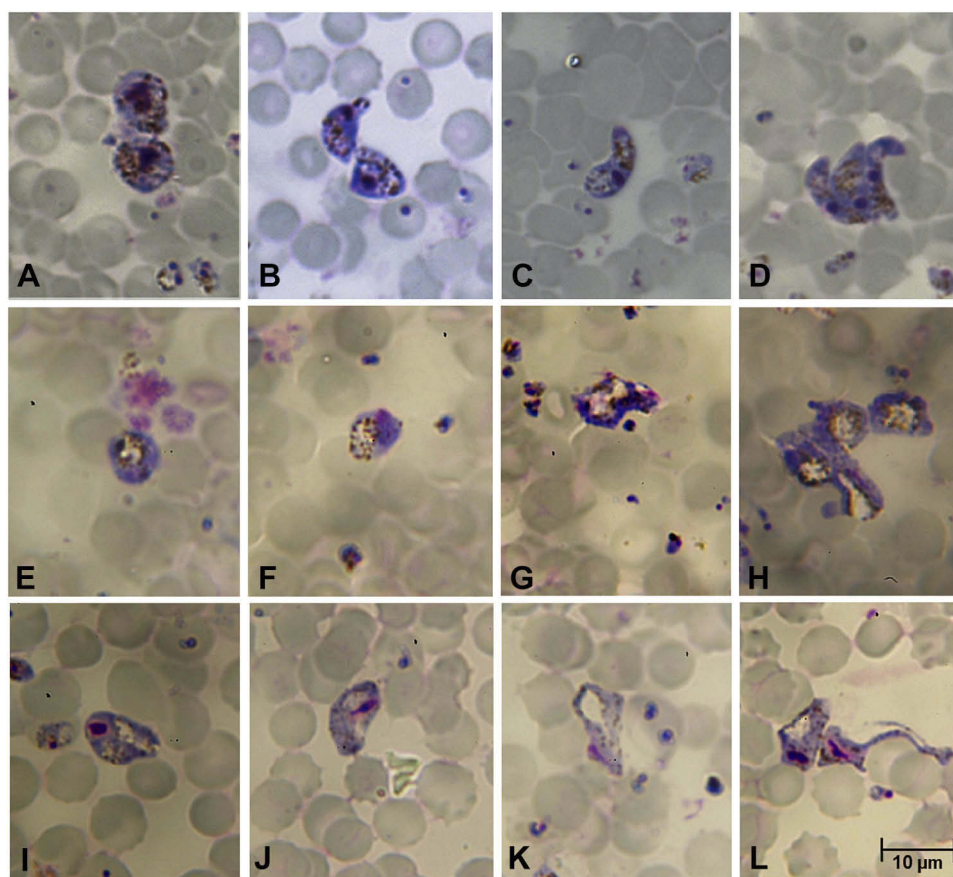
	No. of Elongated Ookinetes	No. of Retort Forms	No. of Zygotes	No. of Altered ESS <sup>a</sup>	Total no. of ESS Forms <sup>b</sup>	% ESS Inhibition
DMSO control	46	30	24	22	122	0
1. MMV000662	0	0	0	2	2	98.4
2. MMV006429	0	0	0	9	9	92.6
3. MMV000642	0	0	0	5	5	95.9
5. MMV665876	0	0	0	34	34	72.0
6. MMV006767	0	1	4	28	33	73.0
8. MMV665827	0	1	1	89	91	25.5

**Notes:** <sup>a</sup>Morphologically altered zygotes and post-zygotic forms as illustrated in Figure 3. <sup>b</sup>Counts were obtained by reading 120 fields at 1000 x magnification at the optical microscope, corresponding to a monolayer of 120 000 to 140 000 red blood cells.

**Abbreviation:** ESS, Early sporogonic stage.

HeatMap,<sup>10</sup> column BH).<sup>30</sup> Slightly better concordance with stage IV–V assays was seen with compound 5 that resulted active in the studies of Duffy & Avery and Taramelli and colleagues (S1 Table. Malaria Box HeatMap,<sup>10</sup> column BO and BP).<sup>30</sup>

Moving along the parasite life cycle to the insect sporogonic development, again activity was found with compounds 1, 2 and 3 (MMV000662, MMV006429 and MMV000642) that inhibited 50% of early sporogonic stages (ESS; including zygotes, retort forms, stumpy and



**Figure 3** Light microscopy images (1000x) from Giemsa stained *P. berghei* early sporogonic stages taken from ODA plates after 24 hrs of incubation in ookinete medium at 19°C. Upper row: images from DMSO control preparations, (A) zygotes, (B) Early retort form, (C) Stumpy ookinete, (D) Fully elongated ookinetes. Middle row: images from Malaria Box compound 5 (MMV665876), (E) Zygote with enlarged hemozoin vacuole, (F) Zygote with enlarged hemozoin vacuole and absence of condensed nucleus, (G and H) Post-zygotic forms with irregular shape, jagged borders, enlarged hemozoin vacuole and absence of condensed nucleus. Bottom row: images from Malaria Box compound 8 (MMV665827), (I–L) Alterations as described in image (G and H) Except for the presence of condensed nuclei.

elongated ookinetes) at 3.1, 39.1 and 40.3  $\mu\text{M}$ , respectively. These results indicate that the compounds interfere with processes occurring before zygote formation, ie with the development of macrogametes, microgametes and/or fecundation. The same compounds have been previously found to be active in the same assay with activities higher than in this study by about a factor of 10 (S1 Table. Malaria Box HeatMap,<sup>10</sup> column from CD to CF).<sup>29</sup> This might be explained by differences in the assay protocol, or in the mouse strain used as gametocyte donor (TO mice by Delves/Sinden, Balb/C mice in this paper) or the method of read out of the fluorescent parasites (plate spectrophotometer by Ruecker et al versus microscope counts at 400x magnification in this study).<sup>29</sup> The plate spectrophotometric read outs appear to overestimate ookinete numbers by about a factor 2 when compared to the Giemsa slide counts. Comparing the results of this study with other literature data, the capacity of compounds 1 and 3 to interfere only with female gamete formation (S1 Table. Malaria Box HeatMap, column BV and BW)<sup>26</sup> and that of compound 2 to impact on female (S1 Table. Malaria Box HeatMap,<sup>10</sup> column BV and BW)<sup>26</sup> and male (S1 Table. Malaria Box HeatMap,<sup>10</sup> column BZ and CA)<sup>29</sup> gamete formation, might have contributed to the reduced ESS counts found in our assays.

At primary screening (50 $\mu\text{g/mL}$ ), compounds 1, 2 and 3 showed more than 80% inhibitory activity specifically on ookinete formation, ie on the process of building up the elongated motile ookinete from the round zygote stage. Similarly, compounds 5, 6 and 8 exhibited more than 70% inhibition against ookinete development. Differential counts on Giemsa thin smears prepared from assay plates (after 24 h incubation with compounds 1, 2, 3, 5, 6, 8) revealed various morphological alterations of post-zygotic forms such as irregular shape, jagged borders, enlarged hemozoin vacuoles and absence of a condensed nucleus. Given this pattern of multiple cellular alterations most likely caused by downstream effects of a primary compound action, it is impossible to raise any hypothesis on the mode of actions. However, what these morphological alterations recall is the biological complexity of ookinete maturation, a process that includes among other the assembly of the apicomplexan organelles and of a cytoskeleton allowing the slender mature ookinete to move, offering multiple targets for possible drug interference.<sup>8</sup>

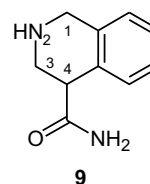
In summary, this work and the results obtained by other studies published in the Van Voorhis review<sup>10</sup> allow to establish for compounds 1, 2, and 3 (MMV000662, MMV006429

and MMV00064) a prominent multi-stage activity against *Plasmodium*: they are active against asexual blood stages, against developing (I–IV) and mature (stage V) gametocytes, they inhibit female gamete formation (also male in the case of compound 2) and interfere with the early sporogonic development, including ookinete maturation. Moreover, this study identified the 1,2,3,4-tetrahydroisoquinoline-4-carboxamide chemical skeleton (9 in Figure 4), including compounds 1–3, as having attractive, fragment-based, druggable chemical properties as reported in previous studies.<sup>18</sup> The 1,2,3,4-tetrahydroisoquinoline skeleton is also encountered in a number of drugs in clinical use such as tubocurarine, nomifensine, diclofenac and quinapril. Due to its distinct geometrical conformation and biological activity, 1,2,3,4-tetrahydroisoquinoline has been introduced into several molecules, which target diverse enzymes or receptors, leading to antineoplastic drugs, nitric oxide (NO) inhibitors, histamine H3 antagonists, serotonin reuptake inhibitors,  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor antagonists, multidrug resistance (MDR) reversals,  $\gamma$ -secretase inhibitors, kinase insert domain-containing receptor (KDR) inhibitors, and antidiabetic drugs.<sup>18</sup>

Regarding antimalarial activity, a recent publication by Delves and colleagues identified various transmission-blocking compounds belonging to the tetrahydroisoquinoline family.<sup>31</sup> In particular, TCMDC-125849 (TCAMS library, GSK) was active in the Pb ODA with an  $\text{IC}_{50}$  of 268 nM and decreased oocyst density in the Pb standard membrane feeding assay by 71.4% at 1  $\mu\text{M}$ . Thus, our results together with the latter findings support the key importance of this skeleton that might allow successful drug development for the control of malaria and various other diseases.

## Conclusion and Recommendation

Thanks to the Malaria Box initiative and the efficient open access policy of MMV, this study was able to contribute to a more comprehensive phenotypic characterization of eight



**Figure 4** 1,2,3,4-tetrahydroisoquinoline-4-carboxamide (9) chemical skeleton, including compounds 1–3, as having attractive fragment-like druggable chemical properties.

antimalarial compounds (1. MMV000662, 2. MMV006429, 3. MMV000642, 4. MMV019266, 5. MMV665876, 6. MMV006767, 7. MMV085583, 8. MMV665827), selected as potential inhibitors of polyamine biosynthesis. Three out of the eight compounds, namely 1. MMV000662; 2. MMV006429; 3. MMV000642, confirmed to possess anti-malarial multi-stage activity, interfering with the parasite asexual blood stages, the gametocytes and the insect early sporogonic stages. Interestingly, this broad spectrum activity appears to remain restricted to the different stages of *Plasmodium* spp.: none of the three compounds was active against the taxonomically related apicomplexan protozoans *Toxoplasma*, *Cryptosporidium*, *Babesia* and *Theileria* spp. Given that the three compounds were also inactive against other protozoan (*Entamoeba*) parasites, various nematodes and *Schistosoma* spp.,<sup>10</sup> these compounds can be excluded as potential multi-disease agents for mass treatment campaigns.

In relation to the recent MMV antimalarial drug policies and attempts to orient research through the conceptualization of Target Product Profiles (TPP) and Target Candidate Profiles (TCP),<sup>4</sup> compounds 1, 2 and 3 fit with the TCP 5 and TCP 6, that focus on transmission-blocking drugs, and TPP 1, that defines drug combinations.<sup>4</sup> In fact, the compounds display an interesting transmission-blocking profile, given by their capacity to interfere with various transmissible stages, in the vertebrate host as well as in the insect vector. This sort of “backed up” transmission-blocking profile enhances the probability to hit a single parasite and hence to achieve interruption of transmission. In addition, given the multi-stage effects of the compounds, that includes activity against the asexual blood stages responsible for the disease, they can be considered potentially valid agents to be included in combination drug formulations. On the basis of the outlined promising activity of compounds 1, 2, and 3, we consider them worth of further structural optimization. Derivative design may be guided by the fact that all the three most active compounds possess a 1,2,3,4-tetrahydroisoquinoline-4-carboxamide chemical skeleton substituted at N-2, C-3 and C-4.

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and *Schistosoma* life stages and assessment of their mechanisms of action” and by the Italian Ministries of Health and of Foreign Affairs/International Cooperation (MAECI), ISARP grant “New generation drugs against *Plasmodium falciparum* transmission for malaria eradication” PGR00948. MCule (<https://mcule.com/>), a pharma and biotech company is acknowledged for providing high-quality MMV Malaria Box Compounds that were used in this study.

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## Author Contributions

HMM, AH and DT conceived the study, coordinated the study and were involved in all stages of the investigation. SDA, SP, SJK and HS carried out the in vitro phenotypic screening of MMV Malaria Box compounds against *Plasmodium falciparum* asexual and gametocyte stages. YAE, ARTG, and HS carried out activity evaluation against early sporogonic development in vitro on *Plasmodium berghei* Pb.CTRPp.GFP strain. All authors contributed to the data interpretation and writing of the manuscript. HMM, AH, DT and SP prepared the final version of the manuscript. All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

## Disclosure

The authors report no conflicts of interest in this work.

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