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A high susceptibility to redox imbalance of the transmissible stages of *Plasmodium falciparum* revealed with a luciferase-based mature gametocyte assay

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Summary

The goal to prevent *Plasmodium falciparum* transmission from humans to mosquitoes requires the identification of targetable metabolic processes in the mature (stage V) gametocytes, the sexual stages circulating in the bloodstream. This task is complicated by the apparently low metabolism of these cells, which renders them refractory to most antimalarial inhibitors and constrains the development of specific and sensitive cell-based assays. Here we identify and functionally characterize the regulatory regions of the *P. falciparum* gene PF3D7_1234700, encoding a CPW-WPC protein and named here Upregulated in Late Gametocytes (*ULG8*), which we have leveraged to express reporter genes in mature male and female gametocytes. Using transgenic parasites containing a *pfULG8*-luciferase cassette, we investigated the susceptibility of stage V gametocytes to compounds specifically affecting redox metabolism. Our results reveal a high sensitivity of

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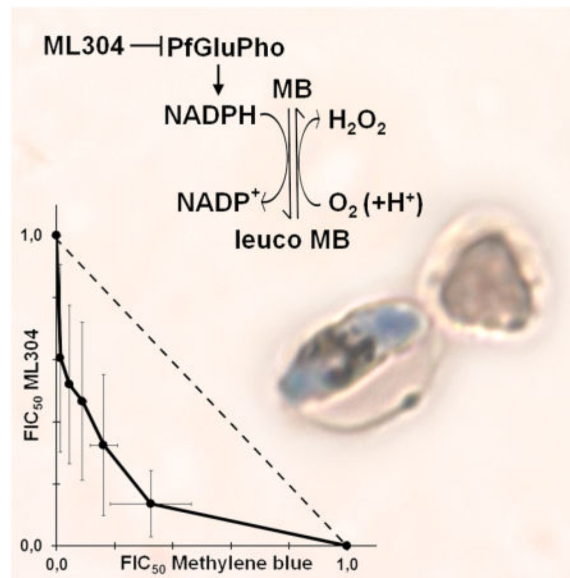
mature gametocytes to the glutathione reductase inhibitor and redox cycler drug methylene blue (MB). Using isobologram analysis, we find that a concomitant inhibition of the parasite enzyme glucose-6-phosphate dehydrogenase-6-phosphogluconolactonase, a key component of NADPH synthesis, potently synergizes MB activity. These data suggest that redox metabolism and detoxification activity play an unsuspected yet vital role in stage V gametocytes, rendering these cells exquisitely sensitive to decreases in NADPH concentration.

ABBREVIATED SUMMARY

The poorly understood physiology of the mature gametocytes of *Plasmodium falciparum* was investigated with bioluminescent parasites, revealing that these stages, known to be refractory to virtually all antimalarial drugs, are exquisitely vulnerable if their ability to contrast oxidative stress is impaired.



Keywords

Plasmodium falciparum; malaria; gametocytes; transmission; methylene blue; transfection; luciferase

Introduction

Malaria is the most prevalent and fatal mosquito-borne parasitic disease. An estimated 3.3 billion people are at risk of being infected by one of the five species of human malaria parasites, of which the most lethal is *Plasmodium falciparum*. In 2015, estimates are that 212 million cases of malaria occurred globally leading to 429,000 deaths, with 92% of the cases in Africa and 70% of the deaths in children under 5 years (WHO World Malaria Report 2016).

The transmission of *Plasmodium* from infected humans to mosquitoes is mediated by sexual stage gametocytes, whose maturation occurs in human red blood cells (RBCs). *P. falciparum*

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gametocytes progress through five stages of maturation (I–V) in ~10 days (Hawking *et al.*, 1971). Immature gametocytes are sequestered in internal organs, including the bone marrow (Joice *et al.*, 2014), and only stage V gametocytes circulate and are transmissible to the mosquito vector during the blood meal.

P. falciparum gametocyte maturation is accompanied by profound physiological and morphological changes including the upregulation of the expression of ~200 gametocyte-specific genes (Young *et al.,* 2005) and proteins (Silvestrini *et al.*, 2010) as well as changes in gametocyte cell-mechanical properties (Tiburcio *et al.*, 2015). The elaboration of subcellular structures and the process of hemoglobin digestion accompany progression of gametocytogenesis from stage I to IV, whereas the final stage of maturation appears to be comparatively less active. Besides the fact that stage V gametocytes persist in circulation or in culture for several days with little, if any, further morphological differentiation, the notion that this stage is comparatively metabolically less active derives from the observation that hemoglobin digestion has ceased (Hanssen *et al.*, 2012) and that most drugs and compounds killing the asexual and the immature sexual stages are no longer active against stage V gametocytes (Adjalley *et al.*, 2011; Plouffe *et al.*, 2016). At present the only exception is primaquine (Smithuis *et al.*, 2010), an approved 8-aminoquinoline recommended by WHO as a gametocytocidal adjunct to first-line artemisinin-based combination therapies (ACT). Primaquine, however, has toxicity issues and can cause hemolysis in individuals harboring certain forms of glucose-6-phosphate dehydrogenase (G6PD) deficiency (Butterworth *et al.*, 2013; Chen *et al.*, 2015).

The apparently lower metabolic activity of *P. falciparum* stage V gametocytes is a major obstacle in the search for novel drugs that can efficiently kill these stages and block malaria transmission. This feature also challenges the development of cell-based drug discovery assays that combine a reliable readout for stage V gametocyte viability with an easy and inexpensive high-throughput screening protocol.

The current notion that stage V gametocytes have a low metabolic activity calls for a deeper investigation of this still obscure aspect of *Plasmodium* biology, an objective that requires sensitive and robust cell-based assays. Of several recently developed assays (Birkholtz *et al.*, 2016), the few specifically designed for *P. falciparum* mature gametocytes are based on high-content imaging of fluorescent gametocytes and gametes or time-lapse imaging of male gamete exflagellation (Ruecker *et al.*, 2014; Lucantoni *et al.*, 2015; Miguel-Blanco *et al.*, 2015).

Here, we leveraged the unsurpassed sensitivity, versatility and scalability of cell-based assays using bioluminescent reporters to produce transgenic *P. falciparum* lines in which a luciferase reporter is driven by a promoter highly upregulated in mature gametocytes. Transcriptomic analyses show that transcript upregulation in mature gametocytes generally occurs through a steady mRNA accumulation rather than an abrupt transcriptional switch from stage IV to stage V (Young *et al.*, 2005; Alano, 2007). Furthermore, several late-stage gametocyte transcripts are not translated until gametes are formed in the mosquito midgut (Paton *et al.*, 1993; Mair *et al.*, 2006). Nevertheless, in this study we report the identification of gene regulatory regions that combine a high level of transcription and a higher expression

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in stage V gametocytes compared to earlier non-transmissible stages. Transgenic parasites engineered to upregulate the expression of a luciferase reporter in stage V gametocytes were used to quantitatively investigate how compounds targeting key pathways of the parasite redox metabolism affected this stage of gametocyte development. This work identified a highly synergistic activity of a redox cycler drug, methylene blue (MB), and a selective inhibitor of the parasite enzyme glucose-6-phosphate dehydrogenase-6-phosphogluconolactonase (PfGluPho), revealing that *P. falciparum* mature gametocytes are highly sensitive to perturbations in redox equilibrium.

Results and Discussion

Identification of *P. falciparum* regulatory sequences upregulating gene expression in stage V gametocytes

Quantitative RNA expression profiles from time courses of *P. falciparum* asexual and sexual development (Le Roch *et al.,* 2003; Young *et al.*, 2005) were examined to select candidate gene regulatory regions that efficiently activated gene expression in stage V gametocytes (Fig. S1). Pairs of upstream and downstream regulatory regions from eight genes whose transcripts accumulated in late stage gametocytes, defined as Upregulated in Late Gametocytes (*ULGs*), were amplified with specific primers (Table S1) and cloned to drive expression of a GFP reporter gene in plasmids p*ULG*-GFP 1 through 8. Flanking regions were also cloned from genes *pfs28* and *mal8p1.16*, the only genomic sequences so far used to upregulate reporter gene expression in late stage gametocytes (Adjalley *et al.,* 2011; Eksi *et al.,* 2008). Each of these ten plasmids (Fig. S2, Table S2) was introduced by electroporation into *P. falciparum* clone 3D7. A preliminary inspection of GFP expression in gametocytes at different stages of maturation showed that in none of the ten transgenic lines was the expression of the GFP reporter specifically restricted to the stage V gametocytes. A quantitative comparison of GFP expression by flow cytometry between synchronous stage II–III and stage V gametocytes from the ten transgenic lines showed that the regulatory regions of gene *pfULG8* outperformed the other *ULG* sequences and the reference genes *pfs28* and *mal8p1.16,* as *pfULG8* regulatory elements yielded a high level of GFP expression in stage V gametocytes. Expression was six–fold higher expression as compared to the immature gametocytes (Fig. 1A; Fig. S3).

Southern blot analysis was performed to compare the gene copy number of the GFP reporter in the 3D7/*pfs28* and in the 3D7/*pULG8-GFP* parasite lines. Intensity of the signal obtained with a GFP-specific probe was similar between these lines, indicating that the higher GFP expression in the 3D7/*pULG8-GFP* gametocytes was not due to *pULG8-GFP* plasmid amplification of but rather to the elevated activity of the *pfULG8* regulatory elements (Fig. 1B).

We then used the *pfULG8* upstream and downstream regulatory regions to drive expression of the *Phyrophorus plagiophtalamus* CBG99 luciferase, which provides exquisite sensitivity (Cevenini *et al.,* 2014). This reporter cassette was integrated into the dispensable *pfcg6* parasite chromosomal locus (Nkrumah *et al.,* 2006), yielding the parasite line NF54-*cg6*-*pULG8*-CBG99 (Fig. S4). Three independent comparisons of luciferase activity between stage V and stage II/III gametocytes of this line showed a 3.8 ± 0.4 higher reporter gene

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expression in the late stages, confirming the ability of the *pfULG8* regulatory sequences to upregulate gene expression in mature gametocytes using an independent reporter that had been chromosomally integrated.

Reporter genes driven by *pfULG8* regulatory sequences escape translational repression and sex-specific expression control but maintain upregulation in stage V gametocytes

The *pfULG8* gene (PlasmoDB ID PF3D7_1234700) is a member of the *P. falciparum* gene family encoding the CPW-WPC proteins. An integrated transcriptomic and proteomic analysis of *P. falciparum* gene expression recently showed that transcripts from the nine CPW-WPC genes predominantly accumulate in female gametocytes and are subject to translational repression (TR) (Lasonder *et al.*, 2016), i.e. these transcripts are produced in gametocytes but are translated only in the mosquito parasite stages. First described for the mRNA of the *Plasmodium berghei* 21kDa ookinete surface protein (Paton *et al.*, 1993), TR was shown to control several rodent malaria gametocyte transcripts through RNA binding proteins recognizing motifs in the mRNA untranslated regions (Mair *et al.*, 2006; Mair *et al.*, 2010). In *P. falciparum*, the TR machinery was reported to rely on interactions of the parasite RNA binding protein Pumilio family 2 (Puf2) with specific sequences in the untranslated regions of transcripts, such as UGUAAUUA or UGUUAAUA in the *pfs25* and *pfs28* mRNAs (Miao *et al.*, 2013).

Our results show that the GFP and the CBG99 luciferase mRNAs regulated by the *pfULG8* and the *pfs28* regulatory sequences are not under TR, as they are efficiently translated in the transgenic gametocytes. This occurs despite the presence of one copy of the TR-responsive motif UGUAAUUA in the *pfULG8* 5′UTR located ~380 nucleotides upstream of the reporter gene start codon, and in the *pfs28* 3′UTR located ~90 nucleotides downstream of the GFP stop codon (Fig. S5). To directly measure whether these transcripts are subject to TR control, GFP fluorescence and CBG99 luciferase activity were measured in the lines 3D7/p*ULG8*-GFP, 3D7/*pfs28*-GFP and NF54-*cg6-ULG8*-CBG99 as they transitioned from mature gametocytes to gametes. Percent of gametocyte ‘rounding up’, measured 15 min after induction, was 85, 80 and 83%, respectively, indicating that efficiency of gamete activation was similar in all lines. Results clearly showed that expression of GFP under the control of either the *pfs28* or the *pfULG8* plasmid-borne regulatory sequences and the activity of the luciferase under the control of the chromosomally-integrated *pfULG8* regulatory sequences were virtually identical before and after induction of gamete formation (Fig. 2A). This confirms that the *pfs28* and *pfULG8* untranslated sequences are unresponsive to TR regulation in the three parasite lines.

It has earlier been proposed that TR regulated mRNAs produced by episomal plasmids might deregulate the TR machinery by titrating components of the TR apparatus (Miao *et al.*, 2013). To examine this possibility in the 3D7/p*ULG8*-GFP and 3D7/*pfs28*-GFP lines, we measured the production of the endogenous Pfs25 gamete/ookinete protein in mature gametocytes and in gametes 6h post-activation. Having observed, as above, no differences in efficiency of gamete activation, the similarly strong increase in Pfs25 protein production during gametogenesis in both the transgenic lines and the non-recombinant parasite lines

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(Fig. 2B) showed that TR is normally functioning and indicated that production of the reporter proteins in gametocytes is not due to a deregulated TR apparatus.

These results are in contrast with the recent report that *pfs28* upstream and downstream untranslated regions mediate a 50–fold increase in the activity of a plasmid-borne firefly luciferase reporter 24h after induction of gametogenesis (Rao *et al.*, 2016). This discrepancy is possibly due to the different time after induction of gametogenesis when reporter activity was measured in the other report (Rao *et al.*, 2016) (24h) and in our experiments (6h). The increase of Pfs25 production 6h after gamete formation (Fig. 2B) clearly indicates that this is an appropriate time to investigate this regulatory mechanism.

To investigate whether escape from TR control of the *pfULG8* driven reporter mRNAs concomitantly affected the female-specific expression of the endogenous *pfULG8* transcript (Lasonder *et al.*, 2016), we labeled 3D7/*pULG8-GFP* stage IV gametocytes with antibodies specific for the female-specific marker Pfg377 (Severini *et al.*, 1999). Two populations of GFP-fluorescent gametocytes were identified, one positive and one negative for Pfg377, respectively present in a 10:1 ratio (Fig. 2C), which clearly indicated that GFP is produced in both female and male gametocytes. The expression of the *pfULG8*-driven GFP in male gametocytes was unambiguously confirmed by observing GFP fluorescence associated with the 3D7/*pULG8-GFP* male gamete exflagellation centers (Movie S1).

Finally, the expression profile of the GFP and the CBG99 reporters under control of the *pfULG8* regulatory sequences was characterized in a daily time course of sexual development of the transgenic gametocytes. GFP fluorescence and bioluminescence of synchronous 3D7/*pULG8-GFP* and NF54-*cg6-ULG8*-CBG99 gametocytes from stage II to stage V were measured for 11 days, confirming the previously observed five-fold increase in the activity of both reporters between stage III (day 4) and stage V (day 11) gametocytes (Fig. 2D).

This analysis in conclusion indicates that the *pfULG8* regulatory regions control the upregulation of a fluorescent or a bioluminescent reporter, maintained as an episomal or a chromosomally integrated construct. The cloned *pfULG8* regulatory regions however no longer mediated female-specific expression and translational repression of the reporter genes, as described for the endogenous *pfULG8* gene, either because these sequences were separated from the natural *pfULG8* locus or because specific motifs were not included in the reporter cassettes. As a result, the *pfULG8* regulatory regions induce production of the reporter proteins in gametocytes of both sexes.

The *pfULG8* driven luciferase reporter as a novel tool to investigate stage V gametocyte physiology

The increased expression of the CBG99 luciferase during late sexual development made it possible to develop a sensitive, quantitative and potentially scalable cell-based assay to investigate the physiology of *P. falciparum* stage V gametocytes. A kinetic analysis of CBG99 bioluminescence produced by the NF54-*cg6-ULG8*-CBG99 stage V gametocytes showed that luciferase activity was stable for at least one hour, with a Coefficient of Variation of 8% between 3 and 40 minutes from substrate addition (Fig 3A), making this

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readout suitable for cell-based drug assays. To validate this assay, we measured the activity of methylene blue (MB), described to kill all gametocyte stages (Adjalley *et al.*, 2011) on NF54-*cg6-ULG8*-CBG99 synchronous stage V gametocytes. Dose-response curves on parasites exposed to MB for 24, 48 and 72h were readily obtained with an excellent assay Z′ factor (Zhang *et al.*, 1999) of 0.88, confirming the potency of MB in killing mature gametocytes and showing that this increased with the time of drug exposure (Fig 3B). Importantly, the assay was able to detect MB gametocytocidal activity after only 24h of drug exposure, which is rarely observed in cell-based assays on stage V gametocytes even after treatment with potent gametocytocidal compounds such as epoxomicin (D'Alessandro *et al.*, 2016).

Alteration of stage V gametocyte redox metabolism

The ability of MB to readily kill stage V gametocytes is intriguing as its mechanism of action in asexual parasites was proposed to act as an inhibitor of *P. falciparum* glutathione reductase (GR; Färber *et al.*, 1998) and as a redox cycling substrate of this enzyme, which uses NADPH to catalyze MB reduction (Buchholz *et al.*, 2008). Although redox activity in gametocytes has been described and exploited in a colorimetric cell-based assay (Tanaka *et al.*, 2011), the low signal to background ratio usually recorded in these assays (e.g. 3, Tanaka *et al.*, 2013) suggests a low level of this metabolic activity in gametocytes.

To gain insights in these processes in mature gametocytes, we tested a panel of inhibitors of several enzymes active in redox metabolism, either alone or in combination with MB for activity against stage V gametocytes. These included uncompetitive inhibitors of both GRs from the parasitized RBCs (M5 in Salmon-Chemin *et al.*, 2001; Davioud-Charvet *et al.*, 2001; Biot *et al.*, 2004; and 3f in Müller *et al.*, 2011), antimalarial NADPH consuming redox cyclers (1c, 3c in Müller *et al.*, 2011 and 17e in Cesar-Rodo *et al.*, 2016), the glutathione depletor paracetamol (Forrest *et al.*, 1982), and one specific inhibitor (ML304) of the bi-functional parasite enzyme glucose-6-phosphate dehydrogenase-6-phosphogluconolactonase (PfGluPho) (Maloney *et al.*, 2012) (Table 1). Dose-response experiments were performed on synchronized late sexual stages of NF54-*cg6-ULG8*-CBG99 with the compounds tested individually or in the presence of MB, with an incubation of 24h to identify fast-acting compounds. Results showed that none of the compounds used alone was active as it failed to produce a dose-response curve or showed an IC_{50} higher than our arbitrarily threshold of 20 μM. In contrast, when tested in combination with MB, the PfGluPho inhibitor ML304 was able to dramatically potentiate MB activity (Table 1). Four independent experiments showed that the fold decrease in MB IC_{50} achieved by 1 μM ML304 was 13.7±6.2.

No decrease in the MB IC_{50} was instead observed if stage V gametocytes were incubated for 24h with 20 μM ML304 and the compound was washed out before an additional 24h treatment with MB alone (data not shown). This indicates that ML304 potentiation of MB activity requires simultaneous presence of both compounds. Further studies showed that ML304, inactive in a 24h treatment (IC_{50} = 56 μM), had noticeable inhibitory activity after longer incubation times (Fig. S6).

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Synergy of MB and the PfGluPho inhibitor ML304 reveals a key role for NADPH production in *P. falciparum* mature gametocyte metabolism

The ability of ML304 to potentiate MB activity against the late sexual stages was further investigated in an isobologram analysis on stage V gametocytes. The IC_{50} values of both compounds, determined in a 48h treatment, were used to design the protocol for the isobologram analysis (Moura *et al.*, 2009). The result of three independent determinations showed a remarkable synergy of MB and ML304 against mature gametocytes (Fig. 4A). The IC_{50} values of MB in the presence or absence of 1 μM ML304 were measured after 2, 6, 12, and 24h of incubation on stage V gametocytes. Results showed that ML304 was able to potentiate MB activity even after a 2h treatment and lowered the IC_{50} value of MB within a nM range after only 12h of co-incubation (Fig. 4B).

These results evoke a consideration of the target of ML304. This compound is a specific inhibitor of the parasite bifunctional enzyme PfGluPho, which catalyzes the first two steps of the pentose phosphate pathway (PPP) that produces >80% of the NADPH in infected erythrocytes, as determined in *P. falciparum* asexual stages (Atamna et al., 1994). PfGluPho is structurally different from the two separate isofunctional human host enzymes and has recently been validated as an antimalarial drug target (Allen *et al.*, 2015). As the redox cycling activity of MB leads to a depletion of intracellular NADPH levels, the most likely explanation for the observed synergy of MB and ML304 is that the consumption of the parasite reducing power caused by MB is augmented by the concomitant inhibition of PfGluPho-dependent NADPH production by ML304 (Fig. 5).

In asexual blood stages NADPH depletion is detrimental, as this molecule is used by the parasite to counteract the oxidative stress caused by hemoglobin digestion and by GR to reduce glutathione disulfide (GSSG) to GSH, which participates in multiple cellular detoxification pathways (Jortzik *et al.*, 2012). In this respect, the potent activity of MB and the synergy with ML304 against stage V gametocytes is surprising as hemoglobin digestion, active in all the immature sexual stages, is reported to cease at this mature stage (Hanssen *et al.*, 2012). This suggests that antioxidant defense depending on reducing equivalents provided by NADPH plays a previously unsuspected crucial role in transmissible gametocytes. Since these parasite stages are metabolically less active than asexual parasites or immature gametocytes a particular susceptibility to oxidative challenge was not anticipated. It will be of great interest to study this phenomenon in more detail by systematically following redox parameters and NADPH levels under various conditions.

Our work supports a re-evaluation of MB as a valuable dual-active drug able to inhibit both asexual parasite growth and mature gametocyte viability in order to meet the urgent need for novel interventions to block parasite transmission. The focus on MB, a clinically licensed drug with nanomolar antimalarial activity (Schirmer *et al.*, 2011), is motivated by several considerations. MB has potent low nanomolar activity against asexual stage parasites (Vennerstrom *et al.*, 1995) and gametocytes *in vitro* (Adjalley *et al.*, 2011; Kasozi *et al.*, 2011), reduces gametocyte carriage rates *in vivo* (Coulibaly *et al.*, 2009), is relatively safe in clinical trials that included G6PD-deficient children (Müller *et al.*, 2013; Meissner *et al.*, 2005; Mandi *et al.*, 2005) and, importantly, has failed so far to select *in vitro* for resistant parasites (D. Fidock, personal communication). MB dual activity against asexual and sexual

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blood stages therefore makes it an excellent candidate as an effective and timely drug that meets the needs of the present malaria elimination/eradication goals and, in the short term, could help address the threat of ACT resistance while new generation anti-transmission drugs are validated and licensed.

This work shows that combining two pro-oxidant compounds such as MB and ML304 effectively inhibits stage V gametocytes within a few hours. Synergy between antimalarial drugs is rarely observed (Co *et al.*, 2009), and this is, to our knowledge, the first to be reported for gametocytocidal compounds. The evidence that potentiation of the MB redox cycler activity significantly increases the inhibition of mature gametocyte metabolism opens the horizon of obtaining novel redox cycler drugs with improved activity, safety and selectivity. This can be achieved by exploring selected compound libraries, by rationally designing ad hoc modifications of the MB chemical scaffolds or by hybridizing the MB phenothiazinium pharmacophore with that of yet to be identified synergistic compounds specific to mature gametocytes.

Experimental Procedures

Inhibitors

Methylene blue trihydrate and paracetamol were purchased from Sigma. The carboxylic acids GR inhibitors M5 (Salmon-Chemin *et al.*, 2001; Davioud-Charvet *et al.*, 2001; Biot *et al.*, 2004) and benzoylmenadione 3f (Müller *et al.*, 2011), plasmodione 1c (Müller *et al.*, 2011), 6-fluoro-plasmodione 17e (Cesar Rodo *et al.*, 2016), and benzoylmenadione 3c (the putative metabolite I of plasmodione) (Müller *et al.*, 2011) were freshly prepared as cited. Anthony Pinkerton, Sanford-Burnham Center for Chemical Genomics at Sanford-Burnham Medical Research Institute, La Jolla, CA kindly provided the compound ML304 (Maloney *et al.*, 2012). Stock solutions of all the compounds were prepared in dimethyl sulfoxide (DMSO) and stored in aliquots at −20°C.

Plasmid construction

The multistep cloning strategies to obtain the p*ULG*-GFP vectors, carrying a Green Fluorescent Protein (GFP) reporter flanked by the *Upregulated in Late Gametocytes (ULG)* regulatory regions (Figure S1) and plasmid PCR2.1-attP-*ULG8*-CBG99 (Figure S2) are described in the Supplementary Data section.

Parasite culture and transfection

The *P. falciparum* 3D7A (Walliker *et al.*, 1987) and NF54^{attB} (Nkrumah *et al.*, 2006; Adjalley *et al.*, 2011) lines were cultured in human 0^+ erythrocytes, kindly provided by Prof. G. Girelli, 'Sapienza' University of Rome, at 5% hematocrit under 5% CO_2, 2% O_2, 93% N_2 (Trager *et al.*, 1976). Cultures were grown in RPMI 1640 medium (Gibco) supplemented with 25 mM Hepes, 50 μg/ml hypoxanthine, 0.25 mM $NaHCO_3$, 50 μg/ml gentamicin sulfate and 10% pooled heat-inactivated O^+ human serum. Ring stage parasites (3–5% parasitemia) were transfected via electroporation with 80–100 μg of plasmid DNA using a BioRad electroporator with 0.31 kV voltage, 960 μF capacitance and resistance set to infinity (Fidock *et al.*, 1997).

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To obtain the transgenic lines expressing GFP under the *ULG, pfs28* and *mal8p1.16* regulatory sequences from episomal plasmids, *P. falciparum* 3D7A was transfected with the appropriate plasmids as described above and selected by adding 2.5 nM WR99210 after an initial growth of 24h in drug-free medium.

The *P. falciparum* parasite line NF54^{attB}, containing a Bbx1 *attB* site in the *cg6* gene (Nkrumah et al., 2006) was used to produce the transgenic line NF54-*cg6-ULG8*-CBG99. The pCR2.1-*attP-ULG8*-CBG99 plasmid (Figure S2) was co-transfected with the pINT plasmid (Adjalley *et al.*, 2011) expressing the *integrase* gene required for stable integration of the CBG99 luciferase-expressing plasmid (Figure S2). Double selection started 24h after transfection by adding 250 μg/ml G418 and 2.5 nM WR99210. After 6 days parasites were treated only with 2.5 nM WR99210 and 3 days later they were allowed to recover in drug-free medium.

Quantification and time course of GFP expression in gametocytes development

Parasites from the ten *P. falciparum* 3D7 transgenic lines expressing GFP under the *ULG, pfs28* and *mal8p1.16* regulatory sequences from episomal plasmids were induced to produce gametocytes by parasite overgrowth. After 48h N-acetyl-glucosamine (NAG) treatment to eliminate residual asexual parasites, stage I/II gametocytes were partially purified from uninfected erythrocytes via 60% Percoll gradient centrifugation (Kariuki *et al.*, 1998) and incubated in complete medium. Aliquots of 10^5 purified stage II/III and stage V gametocytes were collected, centrifuged, resuspended in 200μl of 1× PBS and GFP expression was evaluated by flow cytometric analyses on a FACSCalibur™ instrument (BD Biosciences) using the CellQuest (BD) and FlowJo (Tree Star) software.

In the time course analysis, parasites from the 3D7/*pfULG8-GFP* and 3D7/*pfs28-GFP* lines were induced to gametocyte production as above and aliquots of 10^5 purified gametocytes were collected and analyzed daily for 11 days (from stage II/III to stage V) by flow cytometric analyses on a FACSCalibur™ instrument (BD Biosciences) using the CellQuest (BD) and FlowJo (Tree Star) software.

Quantification of GFP expression and of luciferase activity in stage V gametocytes and in gametes

Gametocytes of the 3D7/*pfULG8-GFP* and 3D7/*pfs28-GFP* lines were produced and purified as above. 10^5 stage V gametocytes were activated to form gametes by adding 20μM Xanthurenic Acid (XA) at room temperature; after 6h GFP expression was quantified by flow cytometry from equal number of stage V gametocytes and gametes.

Gametocytes and gametes at 6h post induction from the NF54-*cg6-ULG8*-CBG99 were obtained as above and aliquots of 10^5 parasites were collected and frozen. Cell pellets were resuspended in 100μl 1× PBS with Complete™ protease inhibitors (Roche); 100μl of BriteLite Plus™ substrate (Perkin Elmer) was added and luciferase activity was measured for 30 seconds on a Lumat LB 9501 Tube Luminometer.

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Western blot analysis

Stage V gametocytes from 3D7 wild-type, 3D7/*pfULG8-GFP* and 3D7/*pfs28-GFP* lines were purified on a Percoll gradient; half of them were activated to transform into gametes and collected 6h after induction. Protein extracts from 10^6 stage V gametocytes and gametes were obtained by lysis in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1 mM PMSF and Complete protease inhibitor Cocktail (Roche) and separated by 4–12% gradient Bis-Tris gel electrophoresis (Novex). Proteins were electroblotted onto a nitrocellulose filter (Protran, 0.2 μm) and membranes were incubated for 1h with the rabbit anti-Pfs25 antibody (MRA-38, from MR4) diluted 1:1000. Horseradish peroxidase-conjugated (HRP) secondary antibodies against rabbit IgGs were diluted 1:30,000 and chemioluminescent reactions were revealed with the Super Signal West Substrate kit (Pierce). The same membrane was saturated with H_2O_2 for 30 minutes and incubated for 1h with the rat anti-Pfg27 antibody (Olivieri *et al.*, 2009) diluted 1:1,000. Horseradish peroxidase-conjugated (HRP) secondary antibodies against rat IgGs were diluted 1:30,000 and chemioluminescent reactions were revealed as above.

Immunofluorescence analysis

3D7/*pfULG8-GFP* stage V gametocytes were fixed on a glass smear with paraformaldehyde 4% – glutaraldehyde 0.075% for 10 minutes and permeabilized with 0.1% Triton X-100 (Tonkin *et al.*, 2004). After 30 min preincubation in 1% BSA, parasites were incubated for 1h with an anti-377B rabbit antiserum (1:800) (Alano *et al.*, 1995). After incubation and washes in 1×PBS, parasites were incubated with affinity purified, rhodamine-conjugated secondary antibody against rabbit IgGs (1:800) and 1μg/ml Hoechst 33258. After final washes, samples were observed in a Leica DFC340 FX camera through a Leica PL FLUOTAR 100× objective in a microscope equipped with filters BP 340–380 (Hoechst), BP 515–560 (rhodamine) and BP 470–490 (fluorescein).

Southern blot analysis

Digestion, blotting and hybridization of parasite genomic DNA with the *cg6* and GFP specific probes are described in the Supplementary Information. To compare the gene copy number of the GFP reporter in the 3D7/*pfs28* and in the 3D7/p*ULG8*-GFP parasite lines we performed Southern blot analysis using *pfcg6*- and a *gfp*- radiolabeled probes (Fig. 1). Genomic DNA was isolated from the parental 3D7 and the recombinant 3D7/*pfs28* and 3D7/p*ULG8*-GFP strains, digested with SpeI and PstI, electrophoresed on a 0.8% agarose gel, and transferred onto a Nytran nylon membrane. Hybridization of the membrane was performed at 54°C with a 150 bp ^{32}P-labeled *gfp* probe that was PCR amplified from the *pfs28*-GFP plasmid DNA using primers #43 and #44 (Table S1). After autoradiography, the membrane was stripped into boiling 0.1% SDS and hybridized at 54°C with a 639 bp ^{32}P - labeled *elo1* probe that was PCR amplified from 3D7 genomic DNA using primers #45 and #46 (Table S1).

Time course of CBG99 luciferase activity in gametocyte development

A NF54-*cg6*-*ULG8*-CBG99 culture was induced to gametocyte production via parasite overgrowth. After 48h N-acetyl-glucosamine (NAG) treatment to eliminate residual asexual

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parasites, stage I/II gametocytes were partially purified from uninfected erythrocytes via 60% Percoll gradient centrifugation (Kariuki *et al.*, 1998) and incubated in complete medium. Aliquots of 10^5 purified gametocytes were collected and frozen daily for 11 days. Bioluminescence of the gametocyte samples was determined as described above.

Luciferase assays on stage V gametocytes

NF54-*cg6-ULG8*-CBG99 cultures induced to produce gametocytes were treated with NAG for 96h, after which gametocytes were purified from uninfected erythrocytes on MACS Separation Columns CS (Miltenyi Biotec) and allowed to mature to stage V over the following 8 days. To calculate IC_{50} values, compounds (Table 1) were serially diluted across ten twofold dilutions and dispensed in 96-well plates in a final volume of 100μl/well. Synchronous 8×10^4 stage V gametocytes were resuspended in 100μl of complete medium and incubated with the compounds at 37°C for the time indicated. To calculate the IC_{50} of MB in the presence of a fixed dose of different compounds, MB was dispensed in 96-well plates as described above. Gametocytes were dispensed as described above with 1 μM of the different compounds and incubated with MB at 37°C. Cell viability was evaluated by adding a non lysing formulation of 0.5 mM D-Luciferin substrate (Cevenini *et al.*, 2014) and measuring luciferase activity for 1 second on a Varioskan™ Flash Multimode Reader (Thermo Scientific). The percent viability was calculated as a function of drug concentration. Curve fitting was obtained by non-linear regression analysis (GraphPad Prism 6.0).

Isobologram analysis

MB and ML304 interactions were assessed over a range of concentrations by a fixed-ratio method based on their IC_{50} values (Moura *et al.*, 2009). For each compound the IC_{50} value was determined after a 48h treatment on mature gametocytes and stock solutions were prepared at 16 times the IC_{50} of each compound. Solutions were combined at MB:ML304 ratios of 10:0, 9:1, 7:3, 5:5, 3:7, 1:9 and 0:10. These starting mixes were serially diluted across ten two–fold dilutions. Cell viability was evaluated by measuring luciferase activity of each sample as described in the Methods section. The percentage of viability was calculated as a function of drug concentration and curve fitting was obtained by non-linear regression analysis (GraphPad Prism 6.0). Fractional IC_{50} values were calculated on the basis of the IC_{50} values obtained per assay for each compounds as described (Moura *et al.*, 2009).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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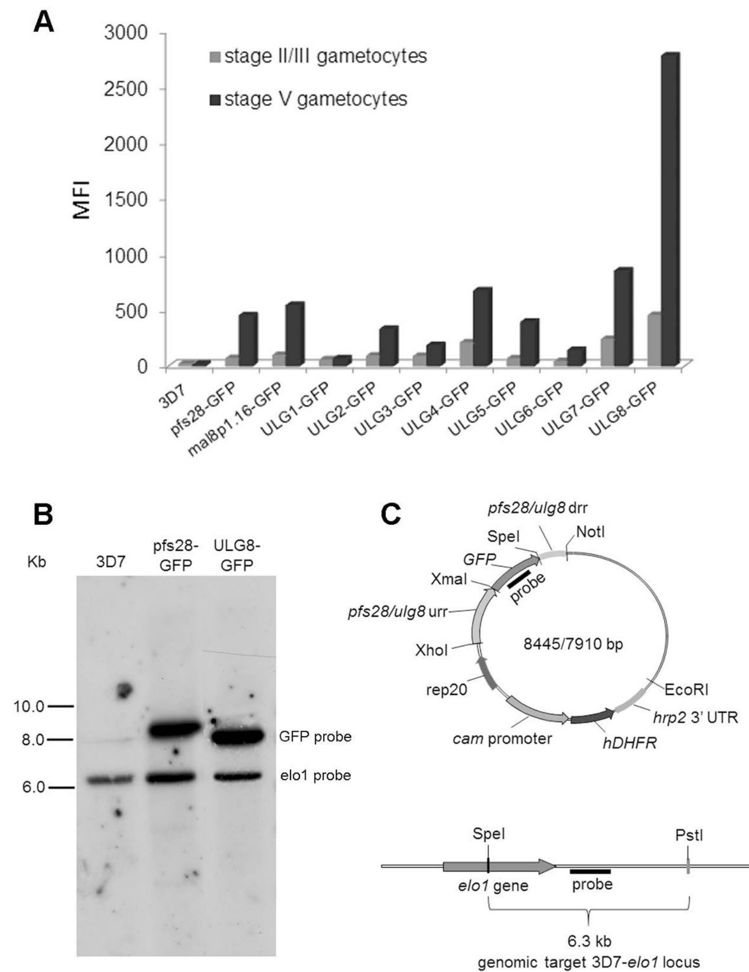

Figure 1. Development of the *P. falciparum* line 3D7/*pfULG8-GFP* upregulating GFP expression in stage V gametocytes

A: Histograms representing the mean fluorescent intensity (MFI) of the GFP reporter expressed under control of the *pfULG 1–8*, the *pfs28* and the *mal8p1.16* regulatory regions in stage II/III and in stage V gametocytes (representative of two biological replicates). B. Southern blot analysis of genomic DNA from lines 3D7wt, 3D7/*pfULG8-GFP* and 3D7/*pfs28-GFP*. Equal amounts of SpeI+PstI digested genomic DNAs from the indicated parasite lines were electrophoresed and hybridized with probes specific for the GFP coding sequence and the single copy gene *pfelo1* (see panel C). Autoradiographs from the two hybridizations were combined in the panel and hybridization bands specific for each probe are indicated. C. Diagram of the plasmid containing the *GFP* coding sequence flanked by the *pfs28* or the *pfULG8* regulatory regions (above) and of the chromosomal locus of the single copy gene *pfelo1* (below) are shown to indicate position of the GFP and the *pfelo1* specific probes, as are the positions of the SpeI and the PstI restriction sites used in the hybridization experiments in panel B. *urr*: upstream regulatory region; *drr*: downstream regulatory region.

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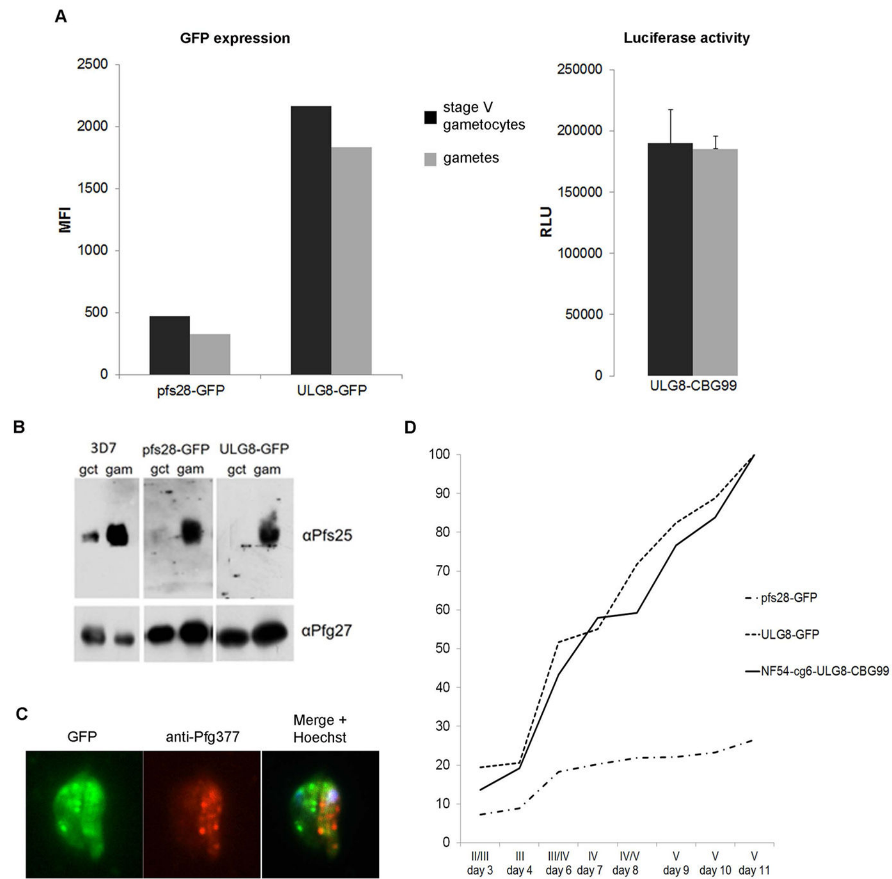

Figure 2. Regulation of reporter gene expression by the *pfULG8* flanking regions

A: GFP and luciferase activity before and after induction of gamete formation. Histograms represent values of mean fluorescence Intensity (MFI, left panel) and of relative light units (RLU, right panel) from gametocytes and gametes 6h post induction of the parasite lines indicated (n = 1). Error bars in RLU histograms represent SD from three technical replicates. B: Translational repression of the Pfs25 gamete/zygote surface protein in wild-type and transgenic parasites. Western blot analysis with Pfs25-specific antibodies on gametocytes (gct) and on gametes 6h post induction (gam) from the indicated parasite lines. Antibodies specific for the gametocyte-specific cytoplasmic protein Pfg27 were used on the same lanes as loading control. C: Staining of 3D7/*pfULG8-GFP* gametocytes with antibodies to the female-specific marker Pfg377. Representative image of two 3D7/*pfULG8-GFP* stage IV gametocytes expressing GFP, one positive to the anti-Pfg377 antibody (female) and one negative (male). D: Time course analysis of GFP fluorescence (MFI) and bioluminescence (RLU) in the course of gametocytogenesis of the indicated lines.

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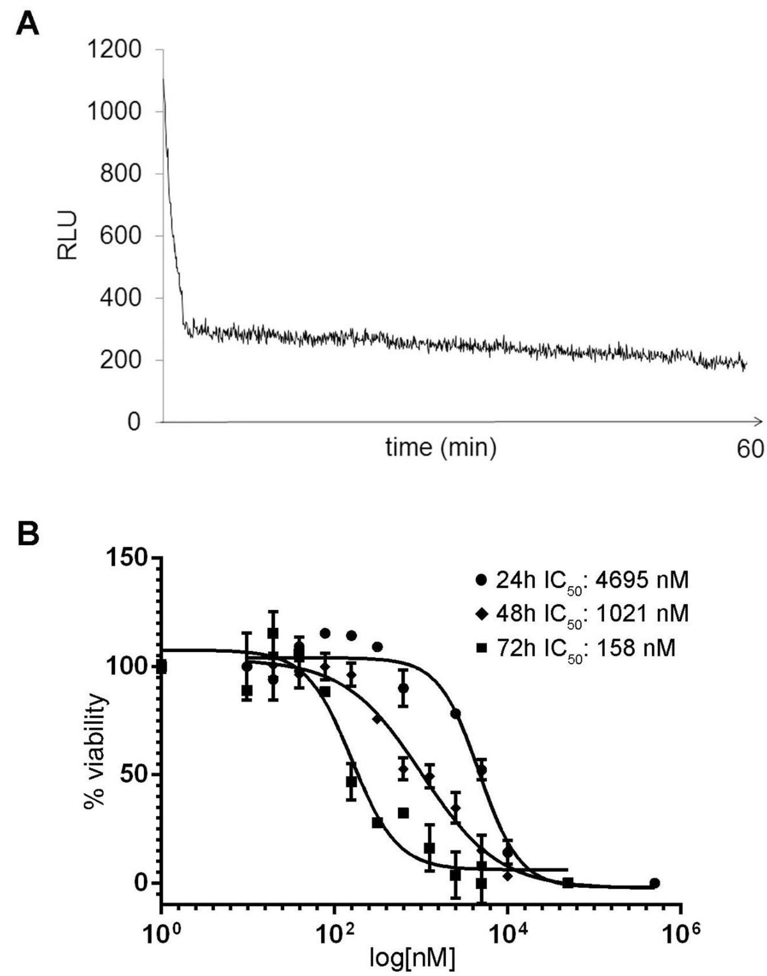

Figure 3. Luciferase reporter activity in NF54-*cg6-ULG8*-CBG99 stage V gametocytes

A: Kinetics of luciferase activity of the CBG99 luciferase reporter produced in stage V gametocytes of the NF54-*cg6-ULG8*-CBG99 line. B: Dose-response curves and IC_{50} values of methylene blue (MB) in 24, 48 and 72h treatments of stage V gametocytes of the NF54-*cg6-ULG8*-CBG99 line.

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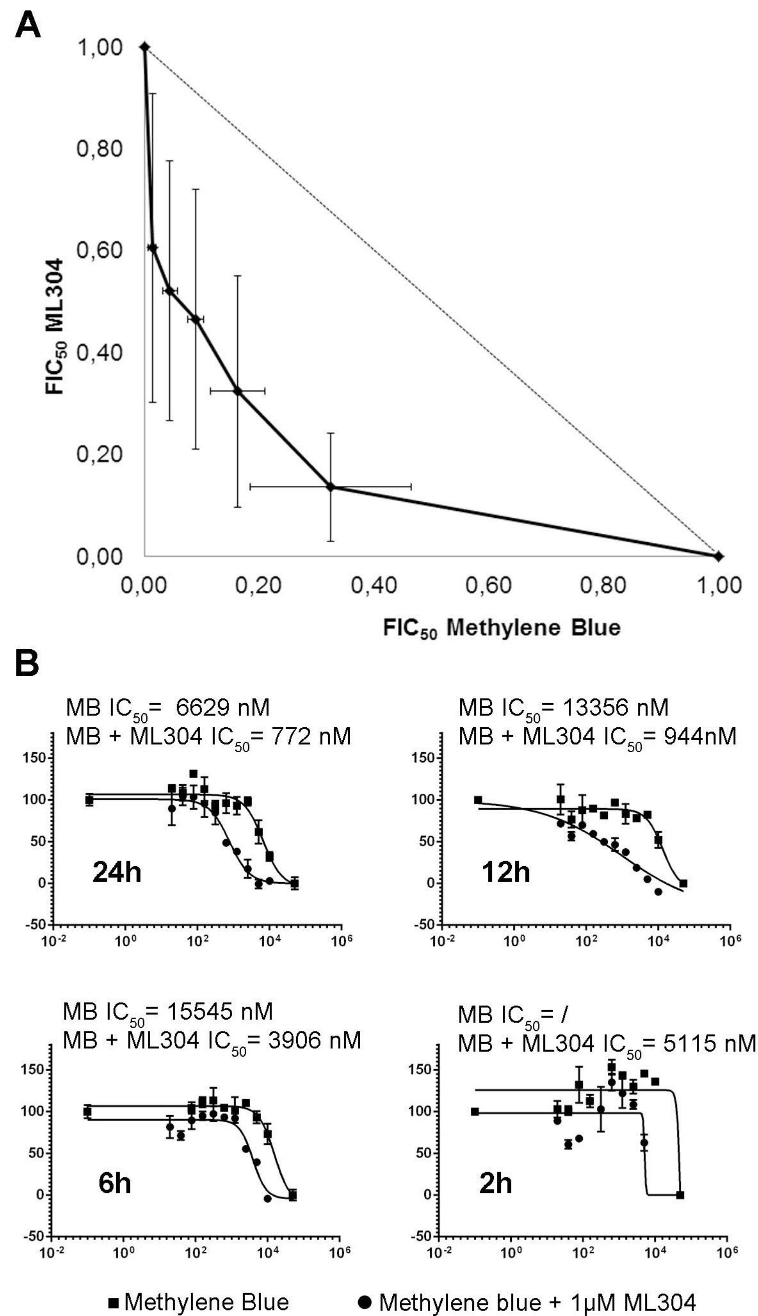

Figure 4. Synergy of MB and ML304 in the inhibition of stage V gametocyte viability

A: Isobologram analysis of the interaction of methylene blue (MB) and ML304 on stage V gametocytes. Determination of the values of the fractional IC_{50} of MB and ML304 is detailed in the Experimental Procedures. Error bars are SD from three independent biological replicates. B: Potentiation of MB activity against stage V gametocytes by ML304. Dose-response curves and IC_{50} values of MB in the absence or presence of 1μM ML304 were determined at decreasing time of drug treatments on NF54-*cg6-ULG8*-CBG99 stage V gametocytes. *x* axes: Log_{10} of inhibitor concentration (nM); *y* axes: percentage viability.

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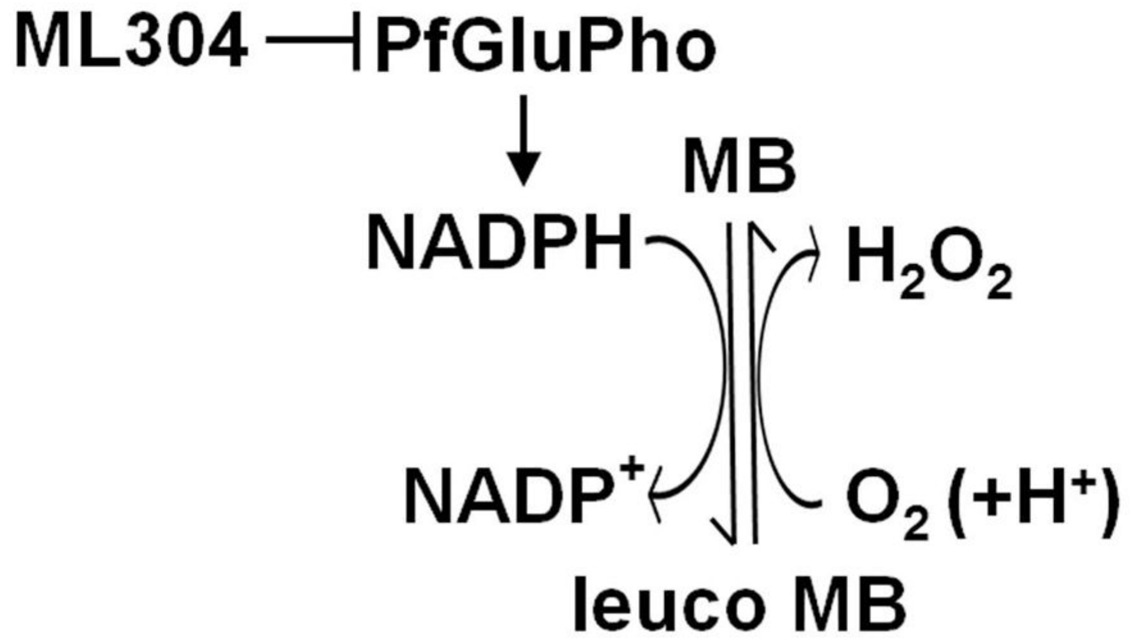

Figure 5. Synergy of MB and the PfGluPho inhibitor ML304 in decreasing NADPH levels

The diagram proposes how the reduction of activity of the parasite enzyme PfGluPho inhibited by ML304 cooperates with the cycles of reduction of MB via the glutathione reductase and spontaneous oxidation in reducing NADPH levels (modified from Buchholz *et al.*, 2008).

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Table 1

Change in MB IC_{50} in 24h treatments of stage V gametocytes in co-incubation with redox perturbing compounds.

Compound code	Description and role on redox	IC_{50} MB alone*/IC_{50} MB + compound (1 μM)	IC_{50} of compound alone
Benzylmenadione 1c (Müller *et al.*, 2011)	Plasmodione (redox cycler and ROS inducer)	0.95	>20μM
Benzoylmenadione 3c (Müller *et al.*, 2011)	Plasmodione metabolite I (redox cycler or GR substrate)	0.95	>20μM
Benzylmenadione 17e (Cesar Rodo *et al.*, 2016)	6-Fluoro- plasmodione (analogue 17e of plasmodione)	0.74	>20μM
M5 (Salmon-Chemin *et al.*, 2001; Davioud-Charvet *et al.*, 2001; Biot *et al.*, 2004)	Menadione derivative (GR inhibitor)	0.60	>20μM
Benzylmenadione 3f (Müller *et al.*, 2011)	Menadione derivative (GR inhibitor)	1.03	>20μM
paracetamol (Forrest *et al.*, 1982)	Acetaminophen (glutathione depletor)	0.41	>20μM
ML304 (Maloney *et al.*, 2012)	(R)-N-((1-ethylpyrrolidin-2-yl)methyl)-4-methyl-11-oxo-10,11-Dihydrodibenzo[b,f][1,4]thiazepine-8-carboxamide (PfGluPho inhibitor)	17.88	>20μM

*The reference IC_{50} for a 24h treatment with MB alone is 4.65 μM.