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Article

Chemogenomic Fingerprints Associated with Stage-Specific Gametocytocidal Compound Action against Human Malaria Parasites

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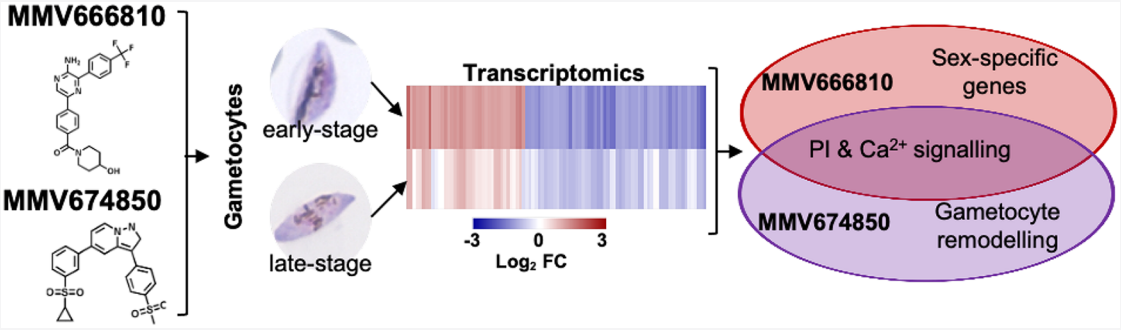

ABSTRACT: Kinase-focused inhibitors previously revealed compounds with differential activity against different stages of *Plasmodium falciparum* gametocytes. MMV666810, a 2-aminopyrazine, is more active on late-stage gametocytes, while a pyrazolopyridine, MMV674850, preferentially targets early-stage gametocytes. Here, we probe the biological mechanisms underpinning this differential stage-specific killing using in-depth transcriptome fingerprinting. Compound-specific chemogenomic profiles were observed with MMV674850 treatment associated with biological processes shared between asexual blood stage parasites and early-stage gametocytes but not late-stage gametocytes. MMV666810 has a distinct profile with clustered gene sets associated primarily with late-stage gametocyte development, including Ca^{2+}-dependent protein kinases (CDPK1 and 5) and serine/threonine protein kinases (FIKK). Chemogenomic profiling therefore highlights essential processes in late-stage gametocytes, on a transcriptional level. This information is important to prioritize compounds that preferentially compromise late-stage gametocytes for further development as transmission-blocking antimalarials.

KEYWORDS: *gametocyte, drug response, kinase, stage-specific, chemogenomic, Plasmodium*

Despite a decade of sustained gains in malaria control programs, the global surge in malaria case numbers in 2017 emphasizes the need for innovative alternative strategies to eliminate the disease.[1] The most lethal form of malaria is caused by *Plasmodium falciparum*, and sustained transmission of this parasite is ensured through human-to-mosquito (*Anopheles* spp.) interaction. As a result, infection is established when the sporozoite forms of the parasite are transmitted to humans and initiate development in the liver, with disease symptoms manifesting after the subsequent release of daughter merozoites and *en masse* infection and rupture of erythrocytes. Intra-erythrocytic development is characterized by multiple and rapid (every ∼48 h) asexual replication cycles. Further onward transmission of the parasite back to a mosquito host is ensured due to the stochastic sexual differentiation of a minor proportion of the asexual parasites into nonreplicative sexual forms, or gametocytes.[2] Gametocytogenesis is an extended process in *P. falciparum* of roughly 10−14 days and is characterized by morphologically and functionally distinct gametocyte stages,[3] with stages I−III grouped as early-stage gametocytes and stages IV−V as late-stage gametocytes. However, only mature stage V gametocytes circulate freely in the bloodstream and are taken up by a mosquito, in which they differentiate into fertile male and female gametes to complete sexual reproduction.

Malaria parasites are targeted by antimalarial compounds that typically either prevent liver stage development when used as prophylaxis or asexual development, thereby alleviating symptoms and preventing death. However, these antimalarials

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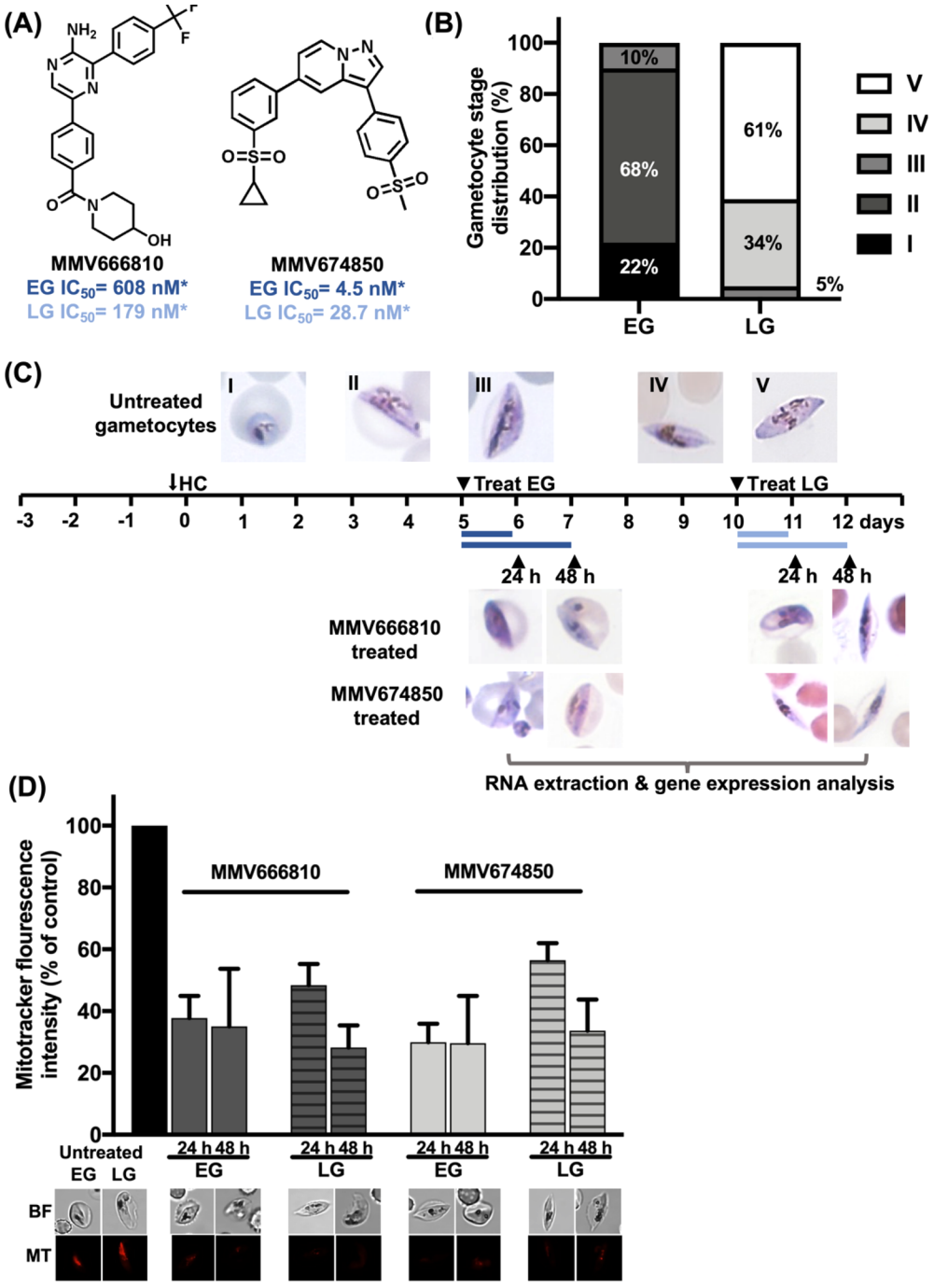

Figure 1. Summary of treatment strategy. Abbreviations indicate early-stage gametocyte (EG), late-stage gametocyte (LG), and hematocrit (HC). *I*–*V* indicates different stages of gametocyte development. (A) MMV666810 (a 2-aminopyrazine) and MMV674850 (a pyrazolopyridine) chemical structures. * = IC_{50} data from ref 12; MMV666810:EG IC_{50} 603 ± 88 nM; LG IC_{50} 179 ± 8 nM; MMV674850: EG IC_{50} 4.5 ± 3.6 nM; LG IC_{50} 28.7 ± 0.2 nM. (B) Gametocyte populations used for drug treatment, classified per stage with Giemsa-stained thin-smear microscopy, calculated by counting ≥100 parasites per condition. (C) Early-stage gametocytes (≥90% stage I and II) and late-stage gametocytes (≥95% stage IV/V) were treated at IC_{90} with MMV666810 and MMV674850 for 24 or 48 h, as indicated, with representative Giemsa-stained thin-smear microscopic pictures of treated gametocytes shown in the bottom panel, compared to untreated gametocytes (top panel). (D) Mitochondrial membrane potential as a viability indicator was measured via Mitotracker red CMXRos staining. The mean fluorescence is indicated as a percentage of the control. Representative brightfield (BF) and mitotracker (MT) fluorescence images shown for each condition.

are usually ineffective at targeting the transmissible gametocyte stages, more often than not due to the underlying biological differences between asexual parasites and sexual gametocytes.[4,5] To contribute to malaria elimination, new chemical entities are required that block the transmission cycle and can be used as add-on drugs to current standard control measures.[1] Currently, only primaquine is approved as an antimalarial able to target gametocytes, but its use is restricted since it can result in hemolytic toxicity in glucose-6-phosphate dehydrogenase deficient patients. Since control strategies cannot rely on a single compound, phenotypic screening efforts are now focused

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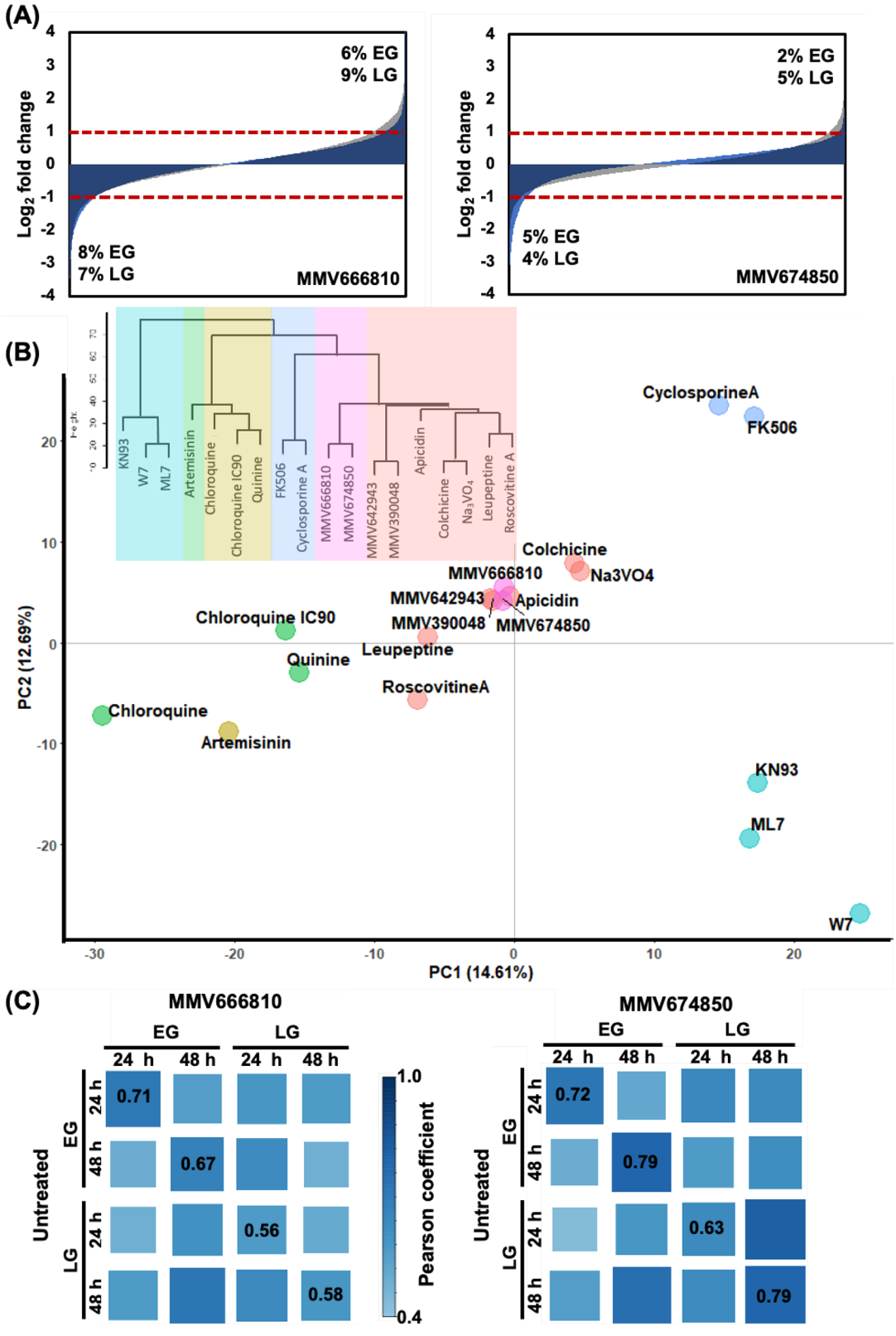

Figure 2. Transcriptomic response of early- and late-stage *P. falciparum* gametocytes treated with MMV666810 or MMV674850. Abbreviations indicate early-stage gametocyte (EG) and late-stage gametocyte (LG). (A) Fold changes after 48 h of treatment compared to the untreated population were ranked by magnitude for both early- and late-stage gametocytes with the percentage of the transcriptome increased or decreased in abundance indicated. Red lines indicate $\log_2$ fold-change thresholds at >1.0 and <−1.0. (B) Principal component analysis (PCA) of 48 h treated MMV666810 or MMV674850 early-stage gametocytes compared to the transcriptomes of asexual parasites following numerous other perturbations,[14] including kinase inhibition by MMV390048 or MMV642943.[12] Dendrogram inset indicates the separation of the compounds. (C) Pearson correlation coefficients calculated for the complete transcriptional profiles of untreated and treated EG and LG as indicated. The size and color intensity of each block indicate the r^2 between the untreated control and the time-matched drug treatment.

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on identifying chemical entities able to target multiple life cycle stages of malaria parasites, including gametocytes.[4−9]

Kinase inhibitors form one of the largest classes of drugs due to their ability to prevent essential phosphorylation of target proteins and disrupt critical cellular signaling systems. Kinase inhibitors are also well described antiplasmodium agents[10] with the 2-aminopyridine, MMV390048, as an antimalarial clinical candidate and a first-in-class inhibitor of *P. falciparum* phosphatidylinositol-4 kinase (PI4K).[11] Multiple members of kinase inhibitor scaffolds have activity against both asexual parasites and gametocytes.[12] However, these inhibitors are largely characterized by compounds that show a preferential ability to target early-stage gametocytes versus late-stage gametocytes.[12] In general, the majority of compounds that are active against asexual stage parasites will retain some activity against early-stage gametocytes but lose activity against late-stage gametocytes completely or are only marginally active against these stages.[12] This pattern holds true for a pyrazolopyridine, MMV674850, with reported activity at 2.7 and 4.5 nM against asexual stage parasites and early-stage gametocytes compared to a 6.4-fold loss in activity against late-stage gametocytes (29 nM).[12] However, deviations from this pattern do occur. For instance, MMV666810, a 2-aminopyrazine similar to MMV390048, is potent against asexual parasites at 5.94 nM, but against gametocytes, it has a 3.3-fold selectivity to late-stage gametocytes compared to earlier stages (179 vs 603 nM, respectively) (Figure 1).[12] The reason for this differential action is unclear, but it may be related to differences in biological target, variable expression levels of the same target in the different stages of gametocytes, or differences in uptake, or it may be ascribed to the underlying biological response to the particular drug pressure in particular developmental stages of the parasite.

Chemogenomic fingerprinting has been associated with drug mode of action (MoA) in *Plasmodium* parasites. Since malaria parasites tightly control transcription at any given point, parasites treated with chemical compounds typically do not have large amplitudes of changes in expression levels.[13] However, compounds with similar MoA do elicit similar responses.[14,15] Interestingly this ability to respond to external perturbation on a transcriptional level is also seen in gametocytes.[12,16,17] Indeed, evaluation of the chemogenomic fingerprint associated with the clinical candidate MMV390048 revealed distinct responses that could explain the functional consequence of inhibiting lipid kinases in the parasite.[12]

Here, we use chemogenomic fingerprinting as a tool to understand the functional consequence of treating early- and late-stage gametocytes with two kinase inhibitors, MMV674850 and MMV666810. For each compound, we obtained distinct global gene expression profiles that explain the differential stage-selective responses observed. Importantly, the late-stage preference of MMV666810 was associated with this compound's ability to affect processes essential to late-stage gametocytes thereby preventing onward gamete formation and transmissibility.

RESULTS AND DISCUSSION

We used global gene expression analysis to interrogate the biological functions that underpin the differential action of two kinase inhibitors, a pyrazolopyridine, MMV674850, and 2-aminopyrazine, MMV666810 (Figure 1A[12]) against early- and late-stage gametocytes. Stage-specific populations of gametocytes were produced as described,[3] determined by morphological analysis of stage-specific features. Here, to assess changes in the transcriptome, early-stage gametocytes (EG, ≥90% stage I and II) and late-stage gametocytes (LG, ≥95% stage IV/V) (Figure 1B) were treated with the two kinase inhibitors, each at the respective IC_{90} for each stage, for 24 and 48 h. These treatments resulted in visible changes in parasite morphology (Figure 1C) and viability as expected (Figure 1D), but there were no statistically significant ($P > 0.05$) differences in viability when comparing the 24 and 48 h time points within a treatment, with viability at 48 h ranging from 28% to 35%.

Kinase Inhibitor Treatment Elicits a Differential Transcriptional Response on Gametocytes. An appreciable change in the transcriptomes was observed following treatment of both early- and late-stage gametocytes treated with MMV674850 and MMV666810. MMV666810 resulted in an increased abundance (log_2 fold-change thresholds at >1.0 and <−1.0 after 48 h) of 6% or 9% of the total transcriptome in early- and late-stage gametocytes, respectively, with a similar proportion of the transcriptome showing decreased abundance (8%). By contrast, MMV674850 causes a lower differential response, with only 2% of early-stage and 5% of late-stage transcripts differentially increased and 5% or 4% of both stages differentially decreased (Figure 2A). These responses are in line with previous results obtained for similar classes of kinase inhibitors against gametocytes.[12] Moreover, a response where ∼10% of the transcriptome is affected is reminiscent of a class of compounds that include calcium/calmodulin-dependent kinase (CDPK) inhibitors (ML7 and W7) and calcineurin when tested against asexual parasites.[14] Importantly, this provides further evidence that the transcriptomes of both early- and late-stage gametocytes respond to external perturbation as we previously reported.[5,12]

A principal component analysis of the drug-induced transcriptional response of early-stage gametocytes following 48 h treatment with either MMV674850 or MMV666810 resulted in a similar grouping with MMV390048 and another 2-aminopyrazine MMV642943 as PI4K inhibitors[12] (Figure 2B, Figure S1). MMV642943 together with MMV666810 are derivatives of 2-aminopyridines, and although they are structurally different from the pyrazolopyridine, MMV674850, their chemogenomic fingerprints are closely related. These kinase inhibitors clearly differentiate into their own cluster (Figure 2B, dendrogram), with the closest other association seen with compounds that causes a general deregulation of the transcriptional cascade including leupeptine and apicidin[14] as seen before.[12] Moreover, the kinase inhibitors used here are either proposed to inhibit or are validated inhibitors of lipid kinases.[11] Therefore, as expected, their chemogenomic signatures are quite distinct from the CDPK inhibitors W7 and ML7 (Figure 2B). This suggests that the transcriptional response to lipid kinase inhibitors is distinct due to the different pathways of intracellular signaling involving lipid kinases or CDPKs.[18,19]

The transcriptional responses induced in gametocytes by MMV674850 or MMV666810 treatment showed limited variability due to treatment time (Figure 2C). Both early- and late-stage gametocytes exhibited similar transcriptomic responses when comparing the 24 to 48 h treatment times, with Pearson correlations of $r^2 > 0.8$ for each drug treatment (Supporting Information). These correlations support the previously seen rate of transcriptional changes evident only after >24 h for kinase inhibitors from these chemical classes.[12] MMV666810 induces the most pronounced differentiation from the untreated control in late-stage gametocytes, with a

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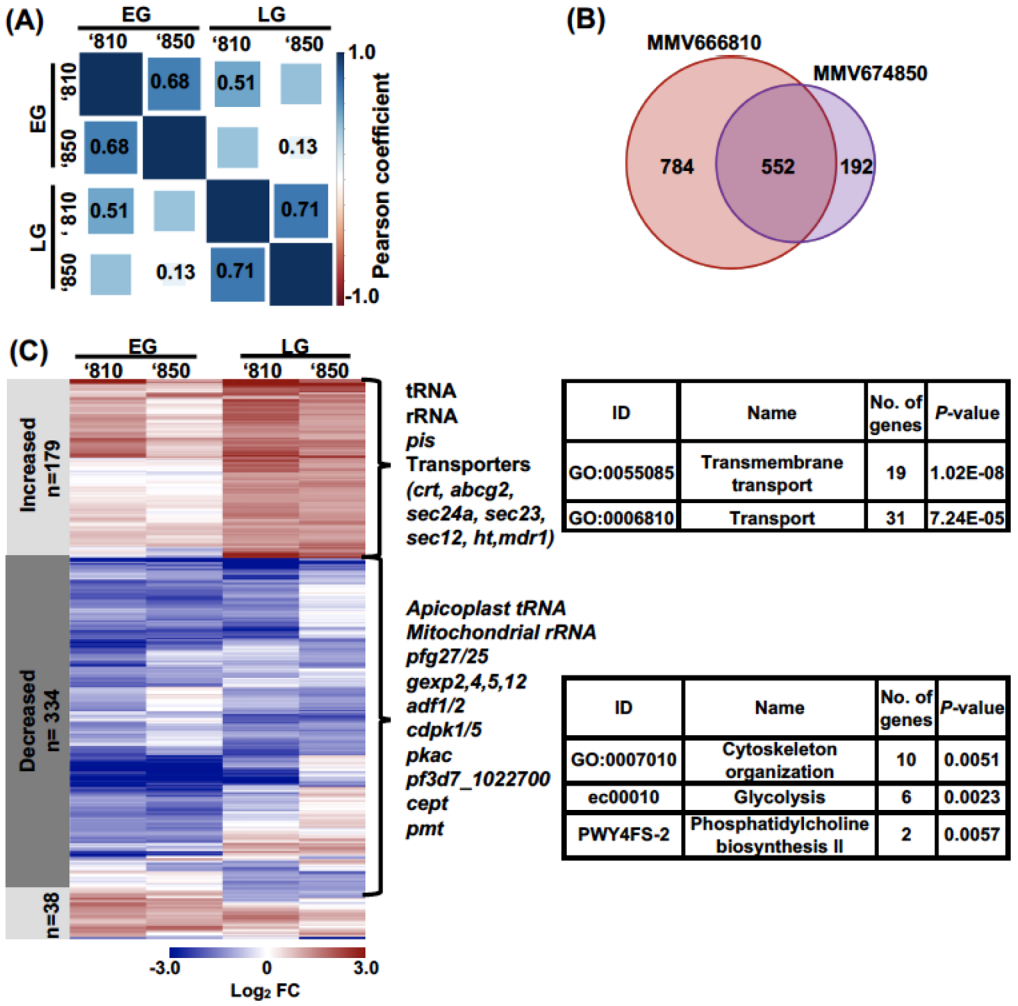

ID	Name	No. of genes	*P*-value
GO:0055085	Transmembrane transport	19	1.02E-08
GO:0006810	Transport	31	7.24E-05

ID	Name	No. of genes	*P*-value
GO:0007010	Cytoskeleton organization	10	0.0051
ec00010	Glycolysis	6	0.0023
PWY4FS-2	Phosphatidylcholine biosynthesis II	2	0.0057

Figure 3. Kinase-specific chemogenomic fingerprint reflects shared biological processes in gametocytes. Abbreviations indicate early-stage gametocyte (EG), late-stage gametocyte (LG), MMV666810 ('810) and MMV674850 ('850). (A) Pearson correlation between early- or late-stage gametocytes treated with either MMV666810 or MMV674850. (B) Venn diagram of all differential transcripts (combined early- and late-stage gametocytes) following 48 h treatment with either MMV666810 or MMV674850. (C) Shared differential transcripts and gene ontology (GO) enrichments in transcripts increased/decreased in abundance following treatment with either MMV666810 or MMV674850. Light gray indicates transcripts with increased abundance; dark gray indicates transcripts with decreased abundance, with full lists available as Supporting Information.

correlation of $r^2 = 0.58$ at 48 h, compared to $r^2 = 0.67$ for early-stage gametocytes, reflecting the preference of this drug for killing late-stage gametocytes. By contrast, such stage-specificity was not as clearly evident for MMV674850.

Shared Kinase Chemogenomic Fingerprint Exhibits Shared Affected Processes in Early- And Late-Stage Gametocytes. We next interrogated the observed shared chemogenomic fingerprint between MMV674850 and MMV666810 (Figure 3). Interestingly, treating either early- or late-stage gametocytes with the different compounds resulted in a more similar profile ($r^2 = 0.68–0.71$) to one another than the overall response between early- and late-stage gametocytes treated with the same compound ($r^2 = 0.13–0.51$) (Figure 3A). This is also reflected in the overlap between differentially abundant transcripts following treatment with each compound (Figure 3B), with most of the transcripts affected following treatment with MMV674850 (74%) also differentially abundant following treatment with MMV666810. This suggests that the stage of gametocyte development relative to the treatment had a greater effect on the cellular response than the compound signature, which is in line with putative lipid kinase inhibitors exhibiting closely related chemogenomic fingerprints (Figure 3B).

Among the transcripts that were increased after treatment with either MMV674850 or MMV666810 were those involved in transport (GO:0006810), similar to the increased abundance of intracellular transport processes reported previously after treatment with MMV390048 or MMV642943.[12] These include genes encoding proteins involved in intracellular trafficking (*sec24a, pf3d7_1361100; sec23, pf3d7_0822600; sec12, pf3d7_1116400; emc2, pf3d7_1410000*), which is analogous to the proposed PI4K-targeting mechanism that dysregulates Golgi membrane trafficking.[18] Strikingly, the transcript for CDP-diacylglycerol-inositol 3-phosphatidyltransferase (*pis, pf3d7_1315600*), the enzyme directly above PI4K in inositol phosphate metabolism (https://mpmp.huji.ac.il/, accessed March 2021[20]), is higher in abundance following treatment with either drug, indicating a possible disruption in inositol phosphate metabolism. Other processes were decreased by treatment with either compound and included cytoskeletal organization (GO:0007010) and glycolysis (ec00010_PK_-KEGG) (Figure 3C). Furthermore, phosphatidyl choline metabolism was also negatively affected (PWY4FS-

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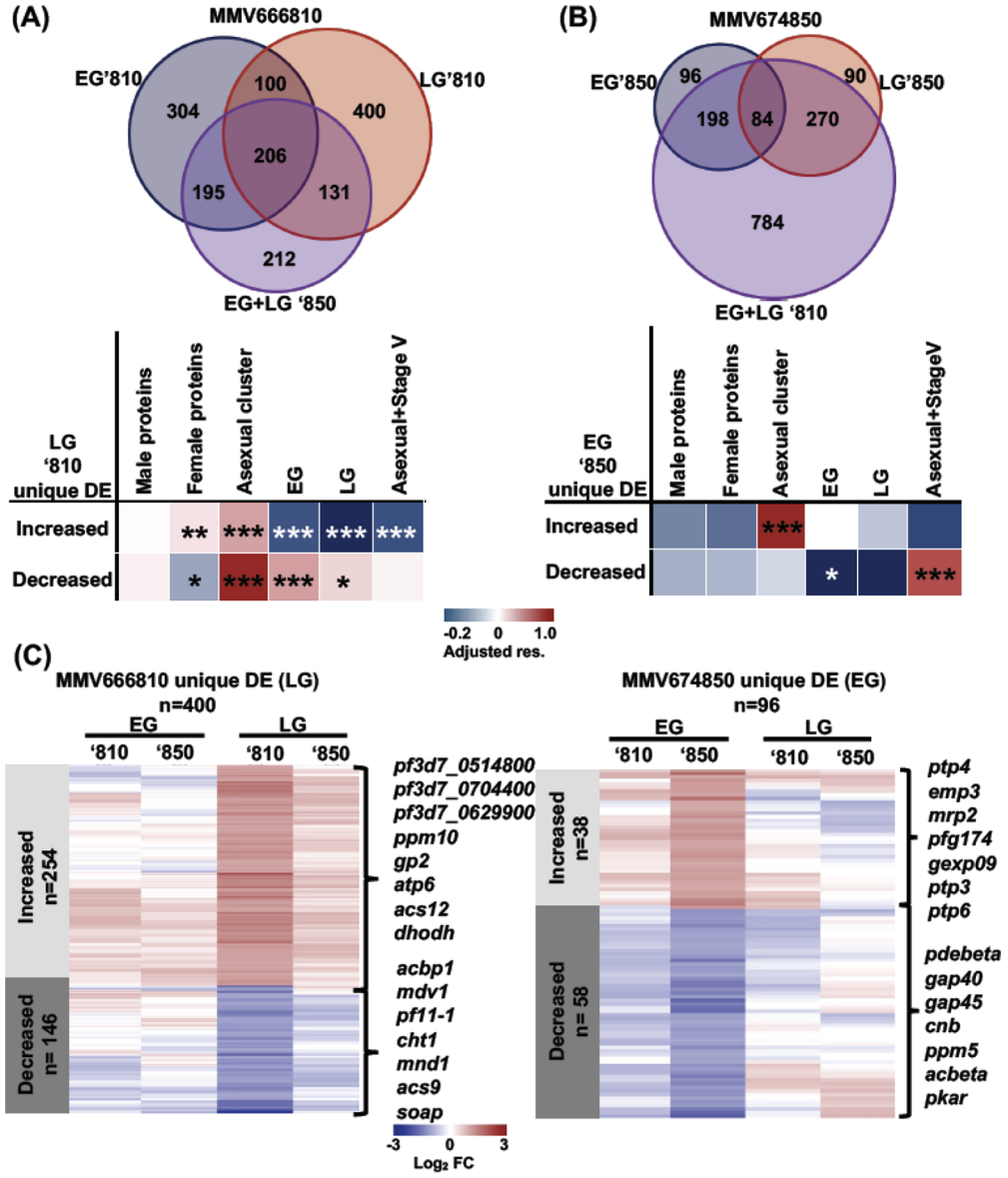

Figure 4. Stage-specific differentiation in gametocyte chemogenomic fingerprint. Abbreviations indicate early-stage gametocyte (EG), late-stage gametocyte (LG), MMV666810 ('810), and MMV674850 ('850), differentially expressed transcripts (DE). Both MMV666810 (A) and MMV674850 (48 h treatment) (B) result in unique chemogenomic profiles based on their transcripts increased or decreased in abundance (Venn diagrams compare early or late-stage transcripts of one compound with the combined early- and late-stage transcripts of the other compound). Adjusted residuals were calculated to illustrate over-representation (RED) or under-representation (BLUE) of sex-specific proteins previously identified in refs 24−26 or genes transcribed at specific points in asexual or gametocyte development previously identified in ref 3 with Fisher exact tests used to determine statistical significance of the overrepresentation (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (C) Euclidean distance clustered heatmaps show uniquely affected transcripts in late-stage gametocyte treatment of MMV666810 or early-stage gametocyte treatment with MMV674850, respectively, with full lists available in Supporting Information.

2__PK__MetaCyc), specifically choline/ethanolaminephosphotransferase (*cept, pf3d7_0628300*), phosphoethanolamine *N*-methyltransferase (*pmt, pf3d7_1343000*), and phospholipid scramblase (*pf3d7_1022700*), suggesting that these compounds also interfere with general phospholipid metabolism. This would be consistent with lipid kinase inhibitors affecting the distribution of lipids in the inner and outer membrane, as PI4K inhibitor MMV390048 results in altered distribution of PI4P between the inner and outer leaflets.[18]

We also noted that numerous exported proteins were among the transcripts with decreased abundance, specifically those exported early in gametocyte development (*gexp2,4,5,12*).[21] The early gametocyte marker *pfg27/25* was also among the most decreased transcripts in any of the compound treatments (Figure 3C). Interestingly, the Ca^{2+}-dependent kinases *cdpk1* and *cdpk5* and actin depolymerization factors *adf1,2* showed decreased transcript abundance following treatment with either compound, but for MMV674850, these transcripts were only decreased in early-stage gametocytes. Gametogenesis critically depends on Ca^{2+} signaling[22] with CDPK1 required for male gamete egress and exflagellation. Moreover, depolymerization of the rigid cytoskeletal network in immature gametocytes is a key feature of gametocyte maturation.[23] Therefore, perturbation of these key regulators of gametogenesis following treatment with MMV666810 compared to MMV674850 at the critical stage of

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sexual development could result in the disparate efficacy profiles of these compounds.

Phenotypic Differences in Stage-Specificity Are Reflected on the Transcriptome. While the transcriptional responses following treatment with either MMV674850 or MMV666810 were largely similar, there were some uniquely affected transcripts (Figure 4). Following treatment of late-stage gametocytes with MMV666810, 400 transcripts were differentially abundant (log_2 fold-change >1.0 increased or decreased) that were not affected following treatment with MMV674850 or upon treating early-stage gametocytes (Figure 4A). To further explore the selective response of either compound, the relative prevalence of stage-specific[3] and sex-specific clusters of genes[24−26] were cross-referenced with the unique differentially abundant transcripts and the significance of these overlaps determined using Fisher's exact tests. This analysis revealed that the differential transcripts in MMV666810-treated late-stage gametocytes were usually transcribed in asexual and/or gametocyte stages, but gametocyte-enriched transcripts were only over-represented in the transcripts with decreased abundance ($P < 0.001$ early gametocyte cluster, $P < 0.05$ in late gametocyte cluster). In addition, transcripts that encode female-specific markers were over-represented in the differentially increased transcripts. Interestingly, this coincides with male markers being decreased following treatment with MMV666810 (male development protein 1 *mdv1, pf3d7_1216500,* meiotic nuclear division protein1 *mnd1, pf3d7_1461500,* and 17 other transcripts including several dynein subunits and dynein-associated proteins coding for male-enriched proteins (Supporting Information), indicating that the late-stage gametocyte specificity may be coupled with deregulation of male gamete development. This effect of MMV666810 on male exflagellation was confirmed, with 80% inhibition of exflagellation observed following a 48 h treatment at IC_{90}. The decreased transcripts also include those necessary for mosquito-stage development (*pf11-1, pf3d7_1038400*[27] *cht1, pf3d7_1252200*[28] *soap, pf3d7_1404300*[29]) and *ap2-sp2* (*pf3d7_0404100*), a regulator of onward sexual replication and sporogony in the murine malaria parasite, *P. berghei.*[30] Therefore, the inhibition of gametocyte maturation observed by MMV666810 treatment of late-stage gametocytes may prevent gametocytes from forming functional, mature stage V gametocytes and subsequently prevent gametogenesis.

While the mRNA encoding CDP-diacylglycerol-inositol 3-phosphatidyltransferase was increased in abundance in every treatment, several transcripts encoding other enzymes in this pathway were only increased in late-stage gametocyte treatment with MMV666810 (inositol polyphosphate multikinase, putative *pf3d7_0514800*, phosphoinositide-binding protein, putative, *pf3d7_0704400*, sec14-like cytosolic factor or phosphatidylinositol/phosphatidylcholine transfer protein, putative, *pf3d7_0629900*) (https://mpmp.huji.ac.il/, accessed March 2021). In addition, genes involved in other closely related processes such as golgi membrane trafficking (Golgi protein 2, *gp2, pf3d7_1123500*) and Ca^{2+}signaling were also affected. This includes a Ca^{2+}-transporting ATPase (*atp6, pf3d7_0106300*) and a putative calcium/calmodulin-dependent protein kinase (*pf3d7_1104900*) responsible for the transport of Ca^{2+} from the cytosol to the endoplasmic reticulum (ER) to establish Ca^{2+} stores in the ER.[31] Ca^{2+} stores in the ER are released as part of the phosphatidylinositol (PI) signaling pathway. Here, the increased abundance of PfATP6 may reflect the cell attempting to compensate for PI4K inhibition that disrupts the phosphatidylinositol signaling pathway by increasing the available ER Ca^{2+} stores. Interestingly, the metabolomic differences after treatment of parasites with MMV390048 resulted in a decrease of peptides produced by hemoglobin digestion, similar to the treatment response of chloroquine[32] and similarly, on the transcriptome level, *pf3d7_1446800*—a transcript encoding for a heme detoxification protein, was decreased in abundance following MMV666810 treatment specifically.

The unique signature produced by treatment of early-stage gametocytes with MMV674850 was notably smaller than for MMV666810, with only 96 transcripts uniquely affected (Figure 4B). Consistent with a selectivity for early gametocytes, the differential transcripts were prevalent in asexual development genes ($P < 0.001$), including genes involved in remodeling either the parasite or erythrocyte membranes. This includes increased transcripts that encode for proteins exported in early gametocyte development (*gexp09, pf3d7_0831000; pfg174, pf3d7_0731300*) as well as proteins that ensure the rigidity of the erythrocyte membrane (*ptp3,4,6*).[33] The transcripts decreased in early gametocytes treated with MMV674850 and were enriched for genes involved in cGMP (phosphodiesterase beta, *pdebeta,* glideosome associated protein 40, 45 *gap40, gap45*), cAMP (adenylyl cyclase beta, *acbeta,* protein kinase A regulatory subunit, *pkar*), and calcium signaling (calcineurin B, *cnb*). Therefore, we conclude that the possible stage selectivity of MMV674850 is due to its preferential effect on erythrocyte remodeling that occurs extensively at the very early stages of gametocyte development.

The complex and diverse biology associated with asexual replication and sexual differentiation of malaria parasites is underscored by a highly regulated and controlled program of gene expression.[3,13] However, the use of functional genomics tools to describe the biological action of or consequences due to antimalarial compound treatment has clearly been established for asexual stage parasites, with metabolic profiling useful as an indicator of phenotypic profile associated with particular chemical classes.[32,34] This also extends to transcriptional profiling where an appreciable and specific chemogenomic fingerprint (on a transcriptome level) can be defined for asexual stage parasites treated with antimalarial compounds.[14,3,35] We demonstrate here that such transcriptional profiles can also be elicited on different stages of gametocytes, confirming our previous evidence that gametocytes can respond to external perturbation on a transcriptional level[12] to allow survival or potentially circumvent the drug effect. The parasite can therefore respond specifically to different classes of drugs, over and above the obvious induction of general stress responses. This implies that the parasite, in its various life cycle stages, may rely on shared processes to respond to drug treatment, aside from the stage-specific stratification in regulatory processes that control gene expression including differentiation in the epigenome,[36] the use of specific transcription factors, or RNA decay.[37]

The ability of the parasite to respond to drug treatment is of course the basis for the development of drug resistance, with the parasite genome being responsive, permissive, and demonstrating plasticity.[38−41] While this information on its own can be useful to understand MoA and identify druggable targets, the transcriptional fingerprint is particularly useful to understand the drug response in gametocytes, as drug targets and/or resistance mechanisms identified in the proliferative asexual

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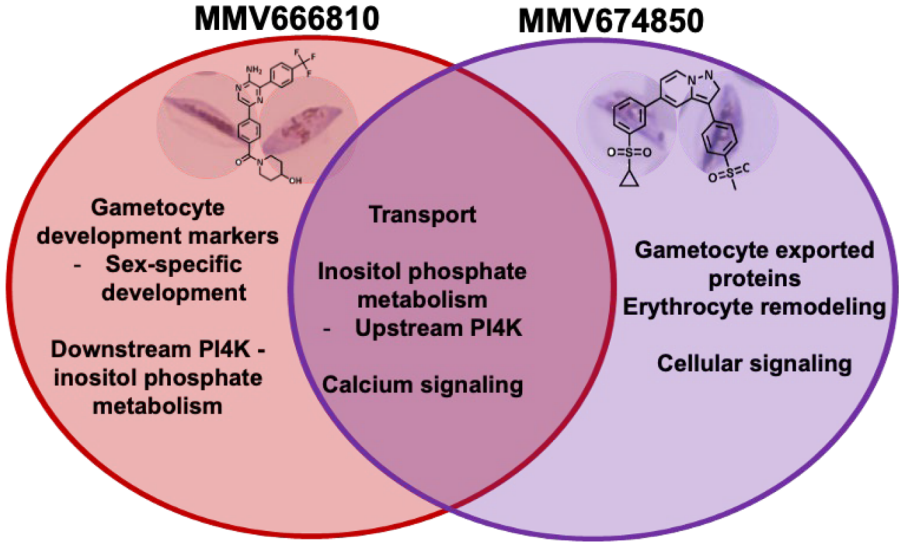

Figure 5. Chemogenomic fingerprints. Both kinase-specific shared biological processes and stage-specific responses revealed.

stage parasites may not be informative toward these non-replicative, differentiated forms. However, it is unclear to what extent the transcriptional fingerprints due to drug treatment in gametocytes can inform risk associated with drug failure and "resistance" formation and if this can be used predictively to indicate possible problems associated with the development of compounds as transmission-blocking antimalarials.

Both early- and late-stage gametocytes are susceptible to lipid kinase inhibition, as seen with MMV390048 and MMV642943[12] and here for MMV674850 and MMV666810. Lipid kinase inhibition results in a shared specific chemogenomic fingerprint with membrane reorganization and lipid metabolic processes affected. Gametocytes extensively remodel the host erythrocyte membrane to meet the requirements of each stage, with more than 10% of gametocyte proteins exported to the host membrane.[21] early-stage intraerythrocytic gametocytes are surrounded by a rigid membrane structure, required for bone marrow sequestration, while deformable stage V gametocytes can clear the spleen and remain available for mosquito uptake.[42] Furthermore, inhibitors of these processes result in a clear fingerprint due to the interruption of the PI and Ca^{2+} signaling pathways and associated Ca^{2+} homeostasis that is unique for lipid kinase inhibitors (Figure 5).[12] This information can be used to target future mechanism of action studies to the specific metabolic pathways identified here, such as inositol metabolism.

The majority of antimalarial compounds with activity against asexual parasites will retain activity on early-stage gametocytes at a comparable level (or with some loss in activity), but those that then do have activity on late-stage gametocytes are typically characterized by an ∼10-fold loss in activity.[5,12] However, evidence is accumulating where compounds are identified with some selectivity toward gametocytes, and with higher potency against these stages than against asexual stage parasites. While this has been shown for diverse chemotypes,[4,5,43,44] it also holds true for compounds with different chemotypes within the kinase inhibitor space.[12,45] Such stage-selective phenotypes have been the topic of much discussion and could be the result of either differences in uptake between asexual stage parasites and gametocytes or differences in target or target availability. Interestingly, a compound like MMV666810 shares some similarity in the core structure with MMV390048, yet it is more potent on late-stage gametocytes compared to early-stage gametocytes. For this single compound, differences in uptake are an unlikely explanation for the potency against gametocytes, with early-stage gametocytes rather being more prone to take up compounds through active new permeability pathways.[46] As MMV674850 treatment affects transcripts involved in erythrocyte remodeling more extensively than MMV666810, it follows that this could correlate with a decreased effectiveness of the latter compound against early-stage gametocytes where this process is integrally important.[21] However, it is known that 2-aminopyridines as well as imidazopyridazines structurally related to MMV674850[46,47] are able to target multiple kinases, and of particular interest is the combination of inhibition of PI4K and also cGMP-dependent protein kinase PKG,[46] as the latter is an essential protein to gametogenesis.[48] While additional fingerprinting studies using compound **2** and ML10 would be needed for absolute confirmation, it is conceivable that the potency seen with MMV666810 in late-stage gametocytes may indeed be due to similar pleiotropic effects where multiple kinases of importance particularly to late-stage gametocytes and gametogenesis are inhibited. Indeed, the transcriptional response elicited by this compound on late-stage gametocytes does severely affect essential processes such as Ca^{2+} signaling that is of importance in male gamete formation and thereby could compromise onward gamete formation and transmissibility. This marks MMV666810 with potential to be exploited as a transmission-blocking antimalarial.

CONCLUSION

In the absence of forward genetic approaches that are typically used to identify drug targets and understand drug mode-of-action in asexual stage parasites, chemogenomic fingerprinting is a valuable tool to expand our understanding of the functional consequence of treating gametocyte stages of malaria parasites, shedding light on the transmission-blocking ability of such compounds.

METHODS

This work holds ethical approval from the University of Pretoria Ethics Committee (EC120821-077) for volunteer donation of

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human erythrocytes for parasite culturing following written informed consent from adult donors only.

Parasite Culturing. Asexual *P. falciparum* NF54 parasite cultures (NF54-*pfs16*-GFP-Luc[49]) were maintained at 5−8% parasitemia, 5% hematocrit at 37 °C in RPMI 1640 medium supplemented as described.[3] The asexual parasites were synchronized with 5% w/v D-sorbitol 6−8 h apart in two consecutive cycles of treatment to obtain >95% synchronicity of ring-stage parasites within a 6 h window. All cultures were maintained with daily medium changes and monitored with Giemsa-stained thin smear microscopy.

Gametocytogenesis was induced via simultaneous nutrient starvation and hematocrit decrease[5,50] by placing ring-stage parasite cultures (>95%) in RPMI 1640 medium prepared as for growth of asexual parasites but without glucose supplementation (day −3) and adjusting the parasitemia to 0.5% and hematocrit to 6%. The hematocrit was reduced to 4% after 72 h (day 0). The medium was replaced after another 24 h (day 1) with normal glucose-supplemented culture medium and gametocytogenesis monitored for a total of 12 days. Contaminating asexual parasites were removed daily with *n*-acetylglucosamine treatment from day 1 to 4 for early-stage gametocytes and from day 4 to 7−8 for late-stage gametocytes.

Parasite Viability. Early (>90% stage I/II) and late (>95% stage IV/V) gametocytes were generated as described above and subjected to microscopic evaluation after treatment with MMV666810 and MMV674850 at IC_{90}. Changes in mitochondrial membrane potential were tracked as a proxy for parasite viability using Mitotracker Red CMXRos dye. After 24 and 48 h, 1 mL of gametocyte culture was recovered and incubated for 30 min at 37 °C in the presence of 30 nM Mitotracker in complete culture medium. Each sample was washed three times with phosphate buffered saline (PBS) and finally resuspended in 500 μL PBS. Brightfield and fluorescent images were obtained using a Zeiss Axioimager microscope and an Axiocam 202 mono digital camera. As a negative control, the gametocytes were treated with 50 μM Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) for the duration of Mitotracker staining. Fiji[51] was used to prepare the final images and obtain the mean fluorescence intensity for each sample.

The exflagellation inhibition assay (EIA) was performed as described previously,[43,52] by capturing the movement of exflagellation centers over time by video microscopy. After 48 h treatment at IC_{90}, a 1 mL aliquot of mature gametocyte culture (>95% stage V) was centrifuged at 3500 rpm for 30 s. The pellet was resuspended in 50 μL ookinete medium (RPMI-1640 medium containing L-glutamine (SIGMA, R6504), 0.024 mg/mL gentamycin (HyClone, SV30080.01), 202 μM hypoxanthine (SIGMA, H9636), 25 mM HEPES (SIGMA, H4034), 0.2% glucose (SIGMA, G6152), 0.5% (w/v) Albumax II (Invitrogen, Paisley, UK) and supplemented with 100 μM xanthurenic acid and 50% (v/v) human serum, A+ male). The induced culture (10 μL) was transferred to a Neubauer chamber at time zero and incubated at room temperature. Movement was recorded by video microscopy (Carl Zeiss NT 6 V/10W Stab microscope fitted with a MicroCapture camera, 10× magnification) and quantified by a semiautomated method.[53] Sixteen videos (8−10 s each) were captured between 16−24 min after incubation. Videos were analyzed using Icy bioimage analysis software.

Microarray Drug Treatment, Sampling, and Hybridization. Both early-stage (>90% stage I/II) gametocytes on day 5 and late-stage (>95% stage IV/V) gametocytes on day 10 were treated at IC_{90} of MMV674850 and MMV666810 and samples removed after 24 and 48 h. Parasite samples (30 mL for untreated cultures and 60 mL drug treated cultures of 2−3% gametocytemia, 4−6% hematocrit) were harvested for both early- and late-stage gametocytes for microarray analysis. The samples removed for early-stage gametocytes were isolated from uninfected erythrocytes via 0.1% w/v saponin treatment for 3 min at 22 °C. Late-stage gametocytes were isolated via density centrifugation using Nycoprep 1.077 cushions (Axis-Shield)[50] centrifuged for 20 min at 800×*g* and the gametocyte containing bands collected.

Total RNA was isolated from each parasite pellet and 3−12 μg total RNA was used for reverse transcription and aminoallyl-dUTP incorporation.[3,12] The reference cDNA pool, coupled to Cy3 consisted of a 1:4 ratio of all the experimental gametocyte samples and cDNA from a 6 hly time course of asexual *P. falciparum* 3D7 parasites. All the other individual samples were coupled to Cy5 dyes (Amersham Biosciences, USA) at pH 9.0 with coupling occurring for 120 min in the dark in an ozone-free environment.[54] Dye coupled cDNA was purified using the Zymo DNA Clean & Concentrator Kit (Zymo Research, USA).

Equal amounts of Cy5 and Cy3 coupled cDNA (150−500 ng) were hybridized to *P. falciparum* custom Agilent 60-mer 8×15k arrays (AMADID#037237),[55,56] in hybridization mixture (1× GE blocking agent, 1XHI-RPM hybridization buffer, Agilent Technologies, USA) for 17 h, rotating at 10 rpm at 65 °C. Before scanning, arrays were washed in first 6× and then 0.06× SSPE, both containing 0.005% *N*-lauryl-sarcosine before being rinsed in acetonitrile. Arrays were scanned on an Agilent G2600D Microarray Scanner (Agilent Technologies, USA) with 5 μm resolution at wavelengths of 532 nm (Cy3) and 633 nm (Cy5). Hybridization and scanning were carried out in an ozone free environment.

Microarray Data Analysis. Within and between array normalization were performed in limma in R using linear lowess normalization. Linear lowess normalized signal intensities were extracted using the Agilent Feature Extractor Software version 11.5.1.1 using the GE2_1100_Jul11_no_spikein protocol and all data uploaded onto the Princeton University Microarray Database (PUMA.princeton.edu). From the signal intensities loaded on PUMA, data were included that had both red and green intensities that were well above background and passed spot filters ($P < 0.01$) and $\log_2$ (Cy5/Cy3) expression values used for further analysis. Quantiles (5th, 95th) of differentially abundant transcripts were calculated in R and guided using a $\log_2$ fold change cutoff of >1.0 or −1.0. Heatmaps were generated using TIGR MeV software version 4.9.0 (http://www.Tm4.org/mev.html). Pearson correlation coefficients were calculated in the R statistical package (version 3.2.3) comparing the expression of genes at each time point with every other time point and visualized using the Corrplot package in R. Fisher exact tests were performed and adjusted residuals calculated in R using verbose code. The differential expression identified in the microarray was confirmed with qPCR performed on the same RNA samples using seryl-tRNA synthetase reference gene (Figure S2). A large-scale comparative data set of drug treatments on asexual parasites[14] and asexual data for MMV390048, MMV642943,[12] as well as data for 48 h of treatment on early-stage gametocytes for MMV666810 and MMV674850 were compiled and cross-normalized through cyclic loess normalization using the limma R package.[57] For each treatment time point, the $\log_2$ fold-change was calculated and time points which showed the most variance, calculated via the

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stats R package, were selected as representatives for the respective treatments. From these time points, an initial PCA was used to determine the number of clusters for conducting hierarchical clustering using the same stats R package. Treatments that contributed to the main cluster/grouping seen in the initial PCA was then visualized in a PCA using the ggplot2 package.

For functional analysis of genes, gene ontology enrichments were obtained for biological processes with $P < 0.05$ using curated evidence from PlasmoDB v33 accessed during Jan 2021 (http://www.plasmodb.org/) and metabolic pathway analysis was performed including both KEGG and MetaCyc databases with $P < 0.01$. Differential gene expression profiles ($\log_2$ fold-change cutoff at >1.0 or <−1.0) for a specific compound treatment at 48 h for either early- or late-stage gametocytes, were associated with clusters of transcripts abundant in either asexual or gametocyte stages of development defined in van Biljon et al.[3] In addition, male or female-partitioned proteins were combined from previously published data sets.[24−26]

The data sets generated for this study can be found in the Gene Expression Omnibus with accession number GSE167068.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.1c00373.

Figure S1: MMV390048 or MMV642943 chemical structures. Figure S2: Verification of microarray-identified differential gene expression with qPCR analyses (PDF)

Additional Excel File provided with a summary of the full gene expression data set with GO and cluster analyses (XLSX)

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Author Contributions

[¶]JN and RvB contributed equally to the work and are shared first authors. RvB, JR, and MvdW performed experiments with assistance by LO. RvB, RvW, AvH, JN, and LMB performed data analyses. JN and LMB conceptualized the study and wrote the paper with RvB, with final editing with ML and KC. All coauthors approved the final version of the manuscript..

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CDPK, calcium/calmodulin-dependent kinase; EG, early-stage gametocytes; LG, late-stage gametocytes; MoA, mode of action; PI4K, phosphatidylinositol-4 kinase

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