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Multistage and transmission-blocking targeted antimalarials discovered from the open-source MMV Pandemic Response Box

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Chemical matter is needed to target the divergent biology associated with the different life cycle stages of Plasmodium. Here, we report the parallel de novo screening of the Medicines for Malaria Venture (MMV) Pandemic Response Box against Plasmodium asexual and liver stage parasites, stage IV/V gametocytes, gametes, oocysts and as endectocides. Unique chemotypes were identified with both multistage activity or stage-specific activity, including structurally diverse gametocyte-targeted compounds with potent transmission-blocking activity, such as the JmjC inhibitor ML324 and the antitubercular clinical candidate SQ109. Mechanistic investigations prove that ML324 prevents histone demethylation, resulting in aberrant gene expression and death in gametocytes. Moreover, the selection of parasites resistant to SQ109 implicates the druggable V-type H+-ATPase for the reduced sensitivity. Our data therefore provides an expansive dataset of compounds that could be redirected for antimalarial development and also point towards proteins that can be targeted in multiple parasite life cycle stages.

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Malaria treatment solely relies on drugs that target the parasite, but current treatment options have a finite lifespan due to resistance development. Moreover, while current antimalarials are curative of asexual blood stage parasitaemia and associated malaria symptoms, they cannot all be used prophylactically and typically do not effectively block transmission. The inability to block transmission is limiting malaria elimination strategies, which requires chemotypes that block human-to-mosquito (gamocyte and gametes) and mosquito-to-human (sporozoites and liver schizonts) transmission.

As few as 100 sporozoites are able to initiate an infection after migrating to the liver for exoerythrocytic schizogony. The subsequent release of thousands of daughter cells, which in turn infect erythrocytes, initiates the extensive population expansion that occurs during asexual replication. A minor proportion (~1%) of the proliferating asexual parasites will undergo sexual differentiation to form mature stage V gametocytes, a 10–14-day process in the most virulent parasite *Plasmodium falciparum*. Only ~10^3 of these falciform-shaped mature gametocytes are taken up by the next feeding mosquito to transform into male and female gametes in the mosquito’s midgut. Fertilization results in zygote development, and a motile ookinite that passes through the midgut wall forms an oocyst from which sporozoites develop, making the mosquito infectious.

The sporozoite and gametocyte population bottlenecks have been the basis of enticing arguments for developing chemotypes able to target them. However, most compounds able to kill asexual parasites are either ineffective in preventing infection and/or blocking transmission or are compromised by resistance development (e.g. antifolates active as prophylactics) or toxicity concerns (e.g. primaquine targeting gametocytes with associated haemolytic toxicity in glucose-6-phosphate dehydrogenase-deficient patients). Patients treated with current antimalarials (or asymptomatic carriers) may harbour enough gametocytes to be transmitted to mosquitoes and sustain the malaria burden. The development of gametocyte-targeted transmission-blocking compounds is therefore essential for a complete strategy directed at eliminating malaria.

Phenotypic screens of millions of compounds have successfully identified antimalarial hits to populate the drug discovery pipeline. However, the majority of these screens assessed activity against asexual blood stage parasites as the primary filter, and hits were only profiled thereafter for activity against additional life cycle stages. While this strategy can identify compounds targeting two or more life cycle stages, it does not allow de novo discovery of compounds with selective activity against specific life cycle stages, such as gametocytes. Parallel screening against multiple life cycle stages would best identify such compounds and rely on selective and predictive assays for gametocytocidal activity, transmission-blocking, and hepatic development. Recently, parallel screening of diversity sets has resulted in reports of such stage-specific compounds, with the benefit that divergent biology associated with the different life cycle stages can be targeted.

In this work, we describe the parallel screen of the Medicines for Malaria Venture (MMV) Pandemic Response Box (PRB) on *Plasmodium* asexual stage parasites, liver stage parasites, mature (stage IV/V) gametocytes, male gametes and oocysts (Fig. 1), as well as mosquito endecticide activity. The MMV, in partnership with the Drugs for Neglected Disease Initiative, assembled the PRB as a collection of 400 drug-like compounds stratified by antibacterial, antiviral or antifungal activity (201, 153, and 46 compounds, respectively), with some compounds having anti-neoplastic activity. The unique and diverse nature of the compounds in the PRB allow one to explore and target the unique biology in the parasite’s different life cycle stages to identify chemical starting points for antimalarial development. All screens were performed on the human parasite *P. falciparum*, except for the liver stages, where the established *Plasmodium berghei* assay was used. Hit selection and progression of compounds in our screening cascade was not biased towards activity on any single life cycle stage, allowing the discovery of multistage-active scaffolds and those with stage-specific activity. Importantly, we report the profiling of a subset of compounds as transmission-blocking molecules that would not have been identified in a test cascade that began solely with an asexual blood stage assay. Four transmission-targeted leads include compounds that are chemically tractable, have favourable physicochemical properties and operate by previously undescribed modes of action, making them amenable to development as transmission-blocking antimalarials.

**Results**

**Parallel screening of the PRB reveals hits against multiple life cycle stages.** To identify active compounds against different stages of the *Plasmodium* life cycle (irrespective of their activity against the other life cycle stages), the PRB was screened in parallel against *Pf* NF54 asexual parasites and stage IV/V gametocytes and *P. berghei* liver stage (Fig. 1). The *Pf* NF54 stage IV/V gametocyte data were orthogonally validated on three independent gametocyte assays to confirm that hit selection (compounds active on at least two platforms) was independent of assay readout. Hits were identified with a relatively lenient but inclusive cut-off of ≥50% inhibition at 2 μM for asexual stages (within 4 S.D.) and 5 μM for gametocytes and liver stages (both within 3 S.D.). Asexual stage activity was confirmed against drug-resistant *Pd*Dd2 asexual parasites. Cytotoxicity filtering was applied after evaluation of the 50% inhibitory concentration (IC50), and transmission-blocking potential of compounds with gametocytocidal activity was confirmed by inhibition of male gamete exflagellation and in a standard membrane feeding assay (SMFA). Assay reproducibility indicators are provided in Supplementary Table 1.

An 18% hit rate was obtained against *Pf* NF54 asexual parasites, 12% against *Pf* NF54 stage IV/V gametocytes and 11% against liver stage parasites (Fig. 2a, Supplementary Data 1). Although a number of compounds showed activity against all these life cycle stages, stage-specific differentiation was evident, as exemplified by the overrepresentation of antifungal compounds in the hit pool for stage IV/V gametocytes compared to asexual parasites (Fig. 2b). The remaining hits reflect the distribution of the compounds in the PRB, with the highest number of hits classified as antibacterials followed by antivirals. The latter seemed to be more potent (as a percentage of the hits) on *Pf* NF54 stage IV/V gametocytes relative to *Pf* NF54 asexual parasites. Only four compounds showed marked toxicity against Chinese hamster ovary (CHO) cells (≤50% viability at 2 μM, Supplementary Fig. 1). Superimposition of the PRB chemical space on current antimalarial drugs within the launched drugs chemical space (Supplementary Fig. 2) indicates little overlap.

Based on the target indicators/biological pathway descriptors for the PRB compounds in other diseases, *Pf* NF54 asexual hits were enriched for inhibitors of kinases, CYP450, energy metabolism and DNA synthesis. Inhibitors of dihydrofolate reductase (DHFR, antifolates), dihydroorotate dehydrogenase, proton pumps and topoisomerase were exclusive hits for *Pf* NF54 asexual and liver stage parasites. Compounds with both *Pf* NF54 asexual and gametocyte activity include antithrombotics, protease inhibitors, sphingosine-1-phosphate receptor modulators and compounds affecting redox homeostasis, whereas inhibitors of the mycobacterial MmpL3 (mycobacterial membrane protein large 3) and ion channels were predominant in gametocyte hits (Fig. 2c). Chemical classes highly represented in the hit pool include quinolines, benzamides/benzoids and azoles.
Multistage-active compounds. All hit compounds were counter-screened against either CHO or HepG2 mammalian cells to remove cytotoxic compounds (Supplementary Data 2) from further consideration for characterization as antimalarials. Of the 72 PRB hits against asexual parasites, all activities were confirmed on re-screening, and of these, 28 compounds passed a more stringent cut-off with IC_{50} values <2 µM (Fig. 2a and Supplementary Data 2), with 16 compounds exclusively active against the asexual stages (Fig. 3a). Of the 51 hits against PfNF54 stage IV/V gametocytes, 18 compounds had IC_{50} values <2 µM. Eight shared activity against asexual stages but ten had gametocyte stage-specific activity (Fig. 3a). Only six compounds showed activity against \textit{P. berghei} liver stages (0.004 \textmu M), with one of these 28 compounds showing activity against both blood stages and liver stages (0.005 \textmu M). Importantly, the majority (13/16) of the asexual-specific chemotypes for further evaluation. Interestingly, the majority of the asexual-specific compounds were classified as antibacterials and tafenoquine (MMV000043), which were both present in the PRB and showed IC_{50} comparable to those previously reported \^\textsuperscript{6} (30 and 940 nM, respectively), validating the screening process (Fig. 4). The most potent of the 28 compounds was an antibacterial diaminoypyridine propargyl-linked antifolate \^\textsuperscript{15}, MMV1580844 (IC_{50} = 0.0017 \textmu M), which targets DHFR in mammalian and yeast cells\^\textsuperscript{16,17}, and was active against \textit{P. berghei} liver stages (0.004 \textmu M) but not PfNF54 stage IV/V gametocytes, supporting previous reports that inhibition of \textit{Plasmodium} DHFR is effective in asexual and liver stages\^\textsuperscript{6}. MMV1580844 had a pronounced (63-fold) loss of activity against pyrimethamine-resistant PfDd2. By contrast, the quinazoline antifolate trimetrexate (MMV1580173, derived from methotrexate) was potently active only against PfDd2 (IC_{50} = 0.108 \textmu M) as well as \textit{P. berghei} liver stages (IC_{50} = 0.0005 \textmu M), in both instances with >10-fold selectivity towards the parasite vs CHO cells.

Asexual-specific chemotypes. Encouragingly, the 28 compounds with asexual parasite activity (Supplementary Data 2) included the known antimalarial compounds chloroquine (MMV000008) and
include two kinase inhibitors, MMV1593539 (IC$_{50}$ = 0.686 μM), a pyruvate kinase inhibitor, and MMV1580482 (URMC-099-C, IC$_{50}$ = 1.3 μM), a mixed lineage kinase 3 (MLK3) inhibitor. Additionally, there is pibenzimol (MMV020752, IC$_{50}$ = 0.149 μM), a disrupter of DNA replication, and MMV019724 (IC$_{50}$ = 1.67 μM), an antiviral lactate dehydrogenase inhibitor.

Liver stage activity is associated with asexual parasite activity. Six dual-stage compounds (asexual blood stage and *P. berghei* liver stage activity ≤2 μM) were identified in the PRB, marking them as having prophylactic potential (IC$_{50}$ range from 0.0005 to 1.72 μM, Fig. 4). These compounds include not only previously described antifolates (MMV1580173 and MMV1580844) but
also the ribonucleotide reductase inhibitor MMV1580496 (triapine) and the bacterial methionyl-tRNA synthetase inhibitor MMV1578884 (REP3123) in addition to the multistage-active compounds, AZD-0156 and Birinapant.

**Unique compounds with stage-specific activity against IV/V gametocytes.** Eighteen compounds were active (IC$_{50}$s < 2 μM) against late-stage gametocytes, as confirmed in at least two of the three orthogonal assays (ATP, PrestoBlue® or luciferase reporter assays, Supplementary Data 2). Of these, eight compounds shared asexual parasite activity, but importantly, ten compounds selectively inhibited PfNF54 stage IV/V gametocytes (>10-fold difference in IC$_{50}$, Fig. 5). The most potent compound was the antineoplastic epidrug ML32418 (MMV1580488, IC$_{50}$ = 0.077 μM), and a marked selection for structurally unrelated inhibitors of G protein-coupled receptors (GPCRs) and related transmembrane proteins were present (Fig. 5a). This includes MMV1581558 (IC$_{50}$ = 0.130 μM) and two MmpL3 inhibitors: the well-characterized 1,2-ethylenediamine antitubercular clinical candidate SQ10919-21 (MMV687273, IC$_{50}$ = 0.105 μM) and a rimonabant derivative MMV158084322 (IC$_{50}$ = 0.108 μM). Two structurally distinct imidazole antifungals also showed potent activity against gametocytes (Supplementary Fig. 3): MMV1634491 (IC$_{50}$ = 0.208 μM) and MMV1634492 (the topical antifungal eberconazole, IC$_{50}$ = 0.23 μM).

**Gametocytocidal compounds target male gametes.** Gametocytocidal hits were validated on male gametes that are generally more sensitive to compounds23. The majority of compounds (13) inhibited male gamete exflagellation by >60% (2 μM, Fig. 5b), 8 of which were potent at ≥80% inhibition. The latter included the gamocyte-targeted compounds MMV1580488 (ML324), the azole antifungals MMV1634491 and MMV1634492 and the MmpL3 inhibitor MMV687273 (SQ109), as well as compounds with additional asexual parasite activity (MMV1580483, MMV396785, MMV1582495, MMV1578570, MMV1581558). The gametocytocidal activity was irreversible for all the compounds except MMV1578570 (Supplementary Fig. 4)24. Six compounds (the epidrug MMV1580488, the two azole antifungals (MMV1634491, MMV1634492), a quinoline MMV1634399 and two MmpL3 inhibitors (MMV1580843, MMV687273)) were directly active on male gametes, implying that shared essential biology between these stages is being targeted.

**Chemotypes with confirmed transmission-blocking activity (TBA).** Transmission-blocking activity was validated by...
the mosquito-based SMFA, using an African malaria vector, Anopheles coluzzii (G3). Transmission-reducing activity (TRA; reduction in oocyst intensity) and TBA (reduction in oocyst prevalence) were determined 8–10 days after feeding female mosquitoes on gametocyte-infected blood, treated with selected compounds (2 µM, Fig. 5c, Supplementary Fig. 5 and Supplementary data 3). Total sample size for the control feeds averaged at 53 mosquitoes, with average oocyst prevalence at 71% and oocyst intensity of 5.8 oocysts/midgut. The TRA for averaged at 53 mosquitoes, with average oocyst prevalence at 71% and oocyst intensity of 5.8 oocysts/midgut. The TRA for

Fig. 4 Asexual blood stage active compounds from the PRB in relation to malaria clinical drugs. PRB compounds active against asexual blood stage (ABS) parasites of P. falciparum. Compounds with inhibitory concentrations (IC50) <2 µM were identified as hits against either PN65 or PfDd2. PRB hits are represented in the launched drugs chemical space (available within the StarDrop V.6.6 software, beige) in comparison to malaria clinical drugs (blue dots). Asexual blood stage actives with IC50 values <2 µM are indicated in red, and gametocyte actives and liver stage actives (all at the same cut-off) are indicated in green and black, respectively, with different dot diameters to highlight compounds active on multiple stages. The 16 asexual-specific compounds are labelled with Compound ID, asexual stage IC50 and structure and other compounds of interest just by name and IC50. CQ chloroquine, TQ tafenoquine.

Endectocide activity. Gametocytocidal compounds were evaluated for their activity as endectocides, killing mosquitoes after supplied in an uninfected blood meal. However, none of the compounds produced significant mortality (one-way analysis of variance (ANOVA), p = 0.7005, total DF = 71, n ≥ 2) in the 4-day mortality assay at 2 µM (Supplementary Fig. 6). Rather, moderate killing (~30%) was observed for the two MmpL3 inhibitors MMV687273 and MMV1580843, the GPCR inhibitor MMV1581558, AZD-0156 (MMV1580483) and MMV1174026, inhibitors MMV687273 and MMV1580843, the GPCR inhibitor MMV1581558, AZD-0156 (MMV1580483) and MMV1174026, marking these compounds with some potential to kill the parasite and mosquito vector.

Mechanistic explorations of transmission-targeted compounds MMV1580488 (ML324) and MMV687273 (SQ109). We investigated the potential mechanism of action of the most potent transmission-targeted compound, ML324, and the antitubercular clinical candidate SQ109.

ML324, together with another inhibitor JIB-04, was recently described as active site inhibitors of a recombinantly expressed
Jumonji-domain containing demethylase (KDM4), jmj3 in Plasmodium29. Inhibition of Pfjmj3 with JIB-04 results in altered histone methylation29, aberrant gene expression and ultimately parasite death. Here we show that ML324 was significantly more potent against late-stage gametocytes compared to early-stage gametocytes and asexual parasites (Fig. 6a). Mechanistically, ML324 treatment significantly increased H3K9me3 levels (a repressive heterochromatin mark in P. falciparum gametocytes)30 in late-stage gametocytes, while not affecting acetylation (Fig. 6b, \( p = 0.028, t \text{ test, } n = 3 \)). This translated to differential expression of 13% of the genome (Fig. 6c, \( p < 0.01, >0.5 \log_2 \text{ fold change (FC) in either direction, Supplementary Data 4} \), similar to
**Fig. 5** Transmission-blocking activity of active compounds from the PRB against *P. falciparum* stage IV/V gametocytes, gametes and oocysts.

a Chemical cluster analysis of the gametocyte hit compounds, using the FragFP descriptor and a Tanimoto similarity index >0.50 in OSIRIS DataWarrior v 5.0.0, and network construction with Cytoscape v 3.7.2. Edges were assigned between similar scaffolds and a parent node. Active compounds with IC$_{50}$ values <2 µM are indicated in green, those with additional activity at the same cut-off on ABS are indicated with hexagons and those with shared activity on liver stages with black borders. Structures are highlighted for selected compounds. Data are from three independent biological repeats (n = 3), each performed in technical triplicates, ±S.E.

b Nineteen compounds with activity against *P. falciparum* stage IV/V gametocytes were evaluated for their ability to inhibit male gamete exflagellation. Compounds (2 µM) were used on stage IV/V gametocytes for a 48-h treatment prior to inducing male gamete exflagellation (carry-over format). Data are from three independent biological repeats (n = 3), performed in technical triplicates, mean ± S.E indicated, except for MMV1633967, MMV1634399 and MMV000043, for which data are from n = 2 biological repeats). Individual data points are indicated in symbols. c SMFA data for 17 compounds (selected based on >50% inhibition on male gamete exflagellation). SMFA was performed by feeding *A. coluzzii* mosquitoes with compound-treated gametocyte cultures (48 h treatment at 2 µM). Data are presented as percentage of TRA (transmission-reducing activity, reduction in oocyst intensity: \( \frac{O_n}{M} = 100 \), where \( i \) oocyst number (intensity), C: control and T: treated) from at least three independent biological repeats (n = 4), performed with technical duplicates, mean ± S.E indicated. Individual data points are indicated in symbols.

**Fig. 6** MMV1580488 (ML324) inhibition of histone demethylation and induction of aberrant gene expression in gametocytes. a Dose–response analysis of ML324 on asexual parasites (ABS) and early- (stage II/III, EG) or late-stage (LG, stage IV/V) gametocytes on the PrestoBlue® assay. Data are from three independent biological repeats (n = 3), performed in technical triplicates, mean ± S.E indicated. 95% confidence intervals on each point indicated as ribbons for each sigmoidal curve. IC$_{50}$ ABS: 2.06 ± 0.119 µM; IC$_{50}$ EG: 0.188 ± 0.029 µM; IC$_{50}$ LG: 0.077 ± 0.001 µM; \(^*\)p = 0.0036 ABS vs LG; \(^*\)p = 0.0052 ABS vs EG, paired, two-tailed t test. b Relative abundance of histone modifications (anti-H3core, anti-H3K9me3 and anti-H3K9ac) for gametocytes treated with ML324 (5 µM) for 24 h. Data are from three independent biological repeats (n = 3), performed in technical triplicates, mean ± S.E indicated. \(^*\)p = 0.028, paired, two-tailed t test. c Transcriptome analysis of gametocytes treated ML324 (5 µM) for 24 h indicating transcripts with significant differential expression (log2 FC > 0.5 increased (red) or decreased (blue) compared to control untreated parasites). GO annotations on biological process level indicated for the increased or decreased abundance gene sets (Fisher’s exact test, two-tailed, exact p values provided on figure per GO term; gene names indicated in Supplementary Data 4).
JIB-04 inhibition of Pfjmj3\textsuperscript{30}. Despite differences in parasite stages evaluated for aberrant gene expression profiles due to JIB-04 or ML324 treatment (asexual vs gametocyte treatments), several processes were similarly affected by these two inhibitors of Pfjmj3 (Supplementary Data 4). Comparatively, there is a complete lack of overlap between aberrant gene expression due to ML324 treatment and that caused by inhibition of a histone methyltransferase with BIX-01294 in gametocytes\textsuperscript{31}, implying at least some conserved and specific responses associated with Pfjmj3 inhibition. ML324 treatment of gametocytes caused repression of known H3K9me3-associated genes involved in cell adhesion (Fig. 6c)\textsuperscript{30} and DNA/chromatin-related processes, including three histone methyltransferases (SET7, 9 and 10), histone H3 and heterochromatin protein 1. Several Api-AP2 transcription factor family members also showed aberrant expression under ML324 pressure including Api-AP2-O3 (ookinetes associated). Among the transcripts with increased abundance is another jmj family member, jmjC230, and gametocyte-associated proteins gametocyte development 1 (gdv1) and male gamete gene 1 (mdv1). The lack of removal of heterochromatic...
H3K9me3 in ML324-treated parasites therefore results in the inability of the parasite to prepare for gametogenesis (concurring with ML324’s efficacy against male gametes, Fig. 5b), since gene silencing is maintained.

SQ109 showed a significant >10-fold selectivity towards late-stage gametocytes compared to asexual parasites ($P = 0.0052$, $n = 3$ paired $t$ test, Fig. 7a). Since SQ109 is an established inhibitor of MmpL3 in mycobacteria with typical rapid action, we evaluated the rate of gametocidal action of SQ109 for 12, 24 and 48 h (Fig. 7b). No significant difference ($P = 0.937$ and $P = 0.558$, respectively; one-way ANOVA, $n = 3$) was observed in the IC50 values, indicating that the effect was present already within 12 h of exposure to gametocytes. Moreover, the activity of SQ109 was similar to the parent compound, SS-ethambutol2HCl (MMV687801.2HCl), whereas a structurally related 2-adamantamine (MMV180402) was inactive (Fig. 7c).

We subsequently evaluated the potential for MmpL3-like targets in P. falciparum. The parasite’s genome does not predict any direct homologues to MmpL3 and only two members of the resistance-nodulation-division (RND)-superfamily (to which MmpL3 belongs) are present. This includes a Niemann–Pick type C1-related H+/lipid symporter (PNCR1)33. However, treatment of a PNCR1 knockdown line with SQ109 did not confer any marked loss of activity, indicating that SQ109 does not likely interact with this protein in Plasmodium spp. (Supplementary Fig. 7). In an attempt to identify alternative molecular targets, resistant mutant selection was performed on P. falciparum 3D7 (A10 clone) under SQ109 pressure. Stable but low-level resistance (2–3-fold increase in IC50 to SQ109 compared to the parental line) was obtained from two independent selections. Whole-genome sequencing of two clones from each selection indicated mutations in the P. falciparum V-type H+-ATPase (PfVpaA, PF3D7_1311900), at one position each: Q225K and R353K (Supplementary table 3). These mutations occur in the A subunit, part of the soluble, ATP-hydrolytic V1 domain of the protein complex. Structural analysis of PfVpaA based on homology (61% sequence identity) to the mammalian V-type ATPase subunit A indicated that both mutations fall within the nucleotide-binding domain of the protein (Fig. 7d–f and Supplementary Fig. 8). Importantly, the Q225K mutation sits within the conserved nucleotide binding pocket of the protein and results in a steric clash with ADP (Fig. 7i), at the catalytic A–B subunit interface, distinguished from the non-catalytic A–B interface (Fig. 7c). PfVpaA has been shown to be present on the parasite’s plasma membrane and digestive vacuole34, and inhibition thereof typically results in parasite death due to disturbance of ATP-dependent H+ efflux and pH regulation35,36.

Discussion

The ability to quickly respond to pandemics has become of paramount importance, and compound sets like the PRB provide an essential tool to support rapid screening of diverse druggable compounds for potential repurposing. Indeed, antimalarials have previously been investigated as antineoplastics37,38 and several antibiotics and antifungals have previously demonstrated anti-malarial activities39,40. Here we screened the PRB across multiple Plasmodium stages and identified chemical matter with antimalarial activity not previously described, providing a useful resource to the research community for drug repurposing as well as lead material for drug optimization.

Multistage activity is a preferential attribute for the next generation of antimalarials41, but such compounds are rarely found in diversity library screens, in large part due to targeted screening approaches rather than parallel screening in multiple assays. We identified two non-cytotoxic multistage-active compounds in the PRB (Birinapant and AZD-0156) that could point to biological parsimony of conserved targets in all these stages, essential to the survival of the parasite such as inhibiting proteins involved in cellular stress responses by either inducing apoptosis or preventing DNA damage recovery responses. Interestingly, Birinapant preferentially killed Plasmodium-infected hepatocytes by reducing host cellular IAP42.

The dual-active asexual and liver stage compounds identified in the PRB have the potential for prophylactic and chemoprotective utility (target candidate profile 4 (TCP-4)), in addition to being chemotherapeutically relevant (target candidate profile 1 (TCP-1))43. Though compounds targeting the same parasite protein in both liver and asexual stages have the associated risk of target-based resistance, the smaller number of parasites in the liver stage reduces this risk. Interestingly targets could be ascribed for some of these TCP-1 and -4 dual-active compounds including MMV1578884 (REP3123) that targets Clostridium difficile methionyl-tRNA synthetase (metRS); to our knowledge there is no aminoacyl-tRNA synthetase (aaRS) inhibitor in clinical antimalarial development, and structural differences between several PfaaRS and their human counterparts leading to selective PfaaRS inhibitors are encouraging43.

The involvement of protein and lipid kinases in key pathogen functions have made inhibitors thereof a focus of drug design strategies including those that affect multiple life cycle stages44. Among the asexual stage active compounds, MMV1580482 (URMC-099) operates as a human MLK3 inhibitor and MMV1593539 as a Staphylococcus aureus pyruvate kinase (PK1) inhibitor, both of interest as kinase targets with a crystal structure for PK1 are available (10.2210/pdb3KHD/pdb), which could guide selectivity and optimization studies, if validated as the target.

Importantly, our parallel screening approach on different life cycle stages yielded compounds and chemical scaffolds that not only have stage-specific asexual parasite activity but also selectively and specifically target the elusive gametocyte stages with activity in mosquito transmission assays. This unbiased approach,
in place of the paradigm where compounds are only profiled for additional life cycle activity once asexual activity has been established, confirms the possibility of identifying gametocyte-specific compounds\textsuperscript{2,8,10}. Indeed, we identified several active compounds that have no former documented antimalarial activity, simply because they were previously not screened against the correct life cycle stage of the parasite, where the relevant biology being targeted was essential.

Our stringent profiling cascade additionally ensured a high success rate in confirming TBA and validates the use of orthogonal gametocytocidal screens\textsuperscript{3,10} as a primary filter in large-scale screens. In addition, a linear correlation between gametocytocidal and male gamete activity was present, which translated to oocyst reduction. By evaluating both TBA and TBA, our data highlighted the importance of both parameters in evaluating SMFA data. Notably, we showed a large reduction in TRA for some compounds; the decreased number of oocysts carried by treated mosquitoes will reduce transmission, as mosquito infection intensity is critically important to the success of transmission\textsuperscript{15}. However, the additional decrease in oocyst prevalence (TBA) implies that the majority of treated mosquitoes would not carry parasites, which could have an epidemiological impact, in line with World Health Organization-recommended vector control interventions.

Gametocyte-targeted compounds would presumably target divergent biological processes compared to asexual parasites\textsuperscript{46}, and this, in addition to the low parasite numbers in transmission stages and non-proliferative nature of gametocytes, would reduce the risk of resistance development. When used in combination with a TCP-1 candidate, such TCP-5 targeted compounds could protect the TCP-1 drug from resistance development. Alternatively, when used as a stand-alone drug, TCP-5 targeted compounds could decrease the gametocyte burden in the human population, which would be particularly important in pre-elimination settings as add-ons to enhance standard measures of malaria control.

Our data indicate specific gametocyte-associated biological processes worthy of further investigation, including two imidazoles (eberconazole, MMV1634492 and MMV1634491). The antimalarial activity of eberconazole, a fungal ergosterol bio-synthesis inhibitor\textsuperscript{47}, has not been described before and may point to an unexplored mode of action. We also identified ML324 with selective activity against early- and late-stage gametocytes, similar to recent reports against immature gametocytes\textsuperscript{29}. As a known Pfmdj inhibitor, we show that ML324 potently kills male gametes with confirmed TBA and does so by preventing histone demethylation, at least for H3K9me as heterochromatin mark\textsuperscript{3}. MmpL3 is also absent in Trypanosoma and Leishmania\textsuperscript{32} and SQ109’s activity is pleiotropic and includes disruption of proton motor forces across membranes as an uncoupler\textsuperscript{32}. Such additional ionophore effects is reminiscent again of additional actions seen for bedaquiline\textsuperscript{34}. These mechanisms are currently being investigated for P. falciparum, including possible disruption of mitochondrial respiration\textsuperscript{46,55} and lipid metabolism/transsucrase which is essential to gametocytogenesis\textsuperscript{36} and oocyst development\textsuperscript{57}.

Our data therefore provide an extensive new set of compounds that could be repurposed or redirected for antimalarial development. One advantage of screening biologically active screening collections such as the PRB is that they contain a large number of compounds with well-described DMPK profiles and empirically determined physical characteristics and that they could progress rapidly through the drug discovery pipeline. Importantly, front-loading screening efforts with biologically active compounds also point towards proteins that can be targeted in multiple parasite life cycle stages.

Methods

Ethics statement. This work holds ethical approval from the University of Pretoria Health Sciences Ethics Committee (506/2018), University of Cape Town: AEC017/026, University of the Witwatersrand Human Research Ethics Committee (M130369) and Animal Ethics Committee (2019/071-70), CSIR Research Ethics Committee (Ref 10/2011) and Scripps Research’s National Blood Donor Service (NBDS), with approval under IRB Number 125933.

Parasite culturing. P. falciparum asexual parasites were cultured in vitro from drug-sensitive strain NF54 (PfNF54), drug-resistant strain Dd2 (PfDd2, chloroquine, pyrimethamine and mefloquine resistant) and the luciferase reporter line NF54-Pf16-GFP-Luc (kind gift from David Fidock, Columbia University, USA)\textsuperscript{58} were used for the various downstream analyses. Gametocytogenesis was induced from asexual NF54-background parasites as described\textsuperscript{5}.

Asexual blood stage screening

Parasite lactate dehydrogenase (pLDH) assay. The asexual blood stage anti-plasmodial activity was assessed on NF54 P. falciparum using the pLDH assay\textsuperscript{59,60}. Initially, all compounds were screened on two separate occasions by seeding ring-stage cultures (1% haematocrit, 2% parasitaemia) in 96-well plates and adding compounds at two concentrations only (2 and 20 µM), and survival was determined after 72 h under an appropriate atmosphere (4% CO$_2$, 3% O$_2$ and 93% N$_2$) at 37 °C, before survival was determined colorimetrically at 620 nm. The anti-plasmodial IC$_{50}$ was determined for suitably active compounds under the same culture conditions as the single-point screens. Chloroquine and artesunate were used as control compounds for all the antiplasmodial assays.

SYBR green I assay. Antiplasmodial activity on drug-resistant Dd2 P. falciparum was determined with SYBR Green I fluorescence\textsuperscript{13}. Briefly, parasite suspension (0.3% parasitaemia, 2.5% haematocrit) was dispensed into 1536-well black, clear bottom plates with pre-spotted compounds using a MultiFluoTM Microplate dispenser (BioTek) at a volume of 8 µL/well. The plates were incubated at 37 °C for 72 h in a low-oxygen atmosphere, after which 10x SYBR Green I (Invitrogen) in Lysis buffer (20 mM Tris/HCL, 5 mM EDTA, 0.16% (w/v) saponin, 1.6% (v/v) Triton X) was added using MultiFluoTM Microplate dispenser (BioTek) at a volume of 2 µL/well. The plates were incubated in the dark at room temperature (RT) for 24 h. The fluorescence was measured by measuring the fluorescence from the bottom of the plates by using the EnVision® Multilabel Reader (PerkinElmer) (485 nm excitation, 530 nm emission). IC$_{50}$ values were determined in CDD vault
P. berghei liver stage assay. Compounds with potential prolymphocytic activity were tested in HepG2-A16-CD81 cells seeded in 1536-well plates (Greiner Bio-One) containing 50 nL of test and control compounds diluted into DMSO and incubated for 24 h in 37 °C. Luciferase activity was determined in 30 μL luciferin substrate (Promega Luciferase Assay System) at RT and semi-automatically quantified with a Promega BacTiter Glo Bioluminescence system4. DHA or methylene blue (MB) were used as positive kill control.

Cytotoxicity counter-screening. CHO toxicity screening. General cytotoxicity of the compounds was determined using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay6 by exposing CHO cells for 48 h at 2 and 20 μM of each compound, and survival was determined colorimetrically. Cells were seeded at a density of 105 cells/well in 200 μL of suitable medium and left overnight (O/N) to attach. After 24 h, medium was gently aspirated from the culture plates, replaced with 200 μL of fresh medium containing the compounds and incubated for a further 48 h. Emetine was used as the control compound for cytotoxicity assessment.

HepG2 toxicity screening. Hepatocellular carcinoma line (HepG2-A16-CD81) was seeded as above for the sporozoite liver stage assay but in the absence of sporozoites. HepG2 toxicity measurements was performed by adding Promega Cell-TiterGlo® (2 μL) followed by luminescence measurement. Curve fitting was done as described above using puromycin (25 μM) as a positive control and DMSO as negative control wells.

Chemical clustering. Drug classes and biological pathways/protein targets were identified based on text and structure searches (PubChem, DrugBank and SciFinder), and chemical space analysis was performed with StarDrop v 6.6 (https://www.collaborativedrug.com/) normalized to maximum and minimum inhibition (DMSO control wells).

Mosquito rearing and SMFA. A. coluzzii mosquitoes (G3 colony, species confirmed in refs. 69, 70) were reared under biosafety level 2 insectary conditions (80% humidity, 25°C, 12 h day/night cycle with 45 min dusk/dawn transition) on a 10% (w/v) sucrose solution diet supplemented with 0.05% 4-aminobenzoic acid. SMFA was conducted using glass feeders (covered with cow intestine/sausage casing to form a membrane) on top of feeding cups (350 mL), maintained at 37 °C. A mature stage V gametocyte culture (1.5–2.3% gametocytaemia, 50% haematocrit in male serum with fresh erythrocytes) was functionally evaluated for male gamete exflagellation and male-female ratio of 1:3 confirmed before proceeding with feeding. The gametocyte culture was either untreated or treated with 2 μM of each compound 48 h. Per glass feeder, 1 mL of the gametocyte culture was added. Each cup contained 25 unfed (2–3 h starvation) A. coluzzii females (5–7 days old), which were allowed to feed in the dark for 40 min. All unfed or partially fed mosquitoes were removed, and remaining females were housed as above for 8–10 days. Mosquitoes were dissected to remove midguts, which were rinsed in phosphate-buffered saline (PBS) and placed into 0.1% (v/v) mercurochrome for 8–10 min and oocysts counted under bright field illumination at ×20–×40 magnification. ATP levels were determined with a Promega BacTiter Glo Bioluminescence system4. DHA or methylene blue (MB) were used as positive kill control.

Histone methylation detection. Late-stage gametocytes were treated with ML324 (5 μM) for 24 h after which histones were extracted from gametocytes for dot blots and enriched as described before69,70. Sampled gametocytes were released from host erythroblast cells using 0.5% (v/v) Triton-X 100 in PBS. The isolated nuclei were washed in PBS to remove residual erythrocyte debris. Nuclei were isolated using a hypotonic lysis buffer (10 mM Tris HCl, pH 8.0, 3 mM MgCl2, 0.25 M sucrose, 0.2% (v/v) Nonidet P-40) with a protease inhibitor (PI) cocktail and then collected by centrifugation for 10 min at 500 × g and 4 °C. This step was repeated and nuclei were resuspended in the same buffer and treated with the nucleases. Restriction enzyme digestion of nuclei were resuspended in a 10 mM Tris buffer (pH 8.0) containing 0.8 M NaCl, 1 mM EDTA and a PI cocktail. Resuspended nuclei were incubated on ice for 10 min and then collected through centrifugation at 10,000 × g for 5 min at 4 °C. Histones were isolated by acid extraction in 0.25 M HCl with rotation at 4 °C O/N. The acid-soluble protein-containing supernatant was retained. The proteinase K digestion was 11,000 × g for 30 min. This was mixed with an equal volume of 20% (v/v) trichloroacetic acid and incubated on ice for 15 min with the pellet collected by centrifugation at 12,000 × g for 30 min. The pellet was washed in acetone, collected again at 12,000 × g for 15 min and then reconstituted in ddH2O. The histone-enriched supernatant was retained following centrifugation at 5000 × g for 2 min. The isolated histones were quantitatively lyophilized and redissolved in an equal volume of nuclease digestion buffer (50 mM NaCl, 1% (v/v) Tween) containing 0% (v/v) overlaid with 5% (v/v) 10 μL of each compound for 48 h at 37 °C. Luciferase activity was determined with a Promega BacTiter Glo Bioluminescence system4. DHA or methylene blue (MB) were used as positive kill control.

DNA microarrays of ML324-treated parasites. DNA microarrays (60-mer, Agilent Technologies, USA) based on the full P. falciparum genome30 that included 5441 annotated transcripts66,67, were performed as before68. P. falciparum NF54 (1–3% gametocytaemia, 4% haematocrit in replicates, stage II/III gametocytes) was

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Data analysis. Assay platforms’ reproducibility was evaluated by Z’-factors (Supplementary Data 1), and data are from a minimum of three independent biological treatment and fold. Statistical evaluation was performed with paired, two-tailed t test for most assays, non-parametric t test (Mann–Whitney) was applied for the SMFA data (GraphPad Prism 8.3.0) and Fisher’s exact two-tailed test for GO annotations. Supra-hexagonal maps were generated in Rstudito 1.1.456 with the RColorBrewer R package.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The authors declare that all relevant data supporting the findings of this study are available within the paper and provided as Supplementary Data files (Files 1–4), supplementary figures (Figs. 1–8) and supplementary tables (Tables 1–3) are included. Transcriptome data is available from GEO (accession code GSE157420), whole-genome sequencing data from NCBI Sequence Read Archive (PRJNA693232) and structural data from Swiss Model Repository (Q76NM6). Source data are provided with this paper.

Code availability

Codes used to analyze data are available at GitHub (https://github.com/Abh-bot/Beehive-inhibition-plots).

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Author contributions
JD, LMB, KC, LLK, GB, EAW and TLC conceptualized the work and supervised the data acquisition. JR, MvdW, DT, NM, SO, AT, PM, SL, BB, EE, NY, LN, AC, NS, JC, DO, EI, LMO performed experiments and data analysis with supervision from KC, EAW, DM, DG, ML, LLK, TLC, CLM, AvH, AH, GB, DC, LLK and LMB performed additional data analysis. LMB, MvW and JR wrote the manuscript with contributions from all authors.

Competing interests
The authors declare no competing interests.