Potent *Plasmodium falciparum* gametocytocidal compounds identified by exploring the kinase inhibitor chemical space for dual active antimalarials

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Running Title: Potent gametocytocidal kinase inhibitors against malaria

SYNOPSIS

Objectives: Novel chemical tools to eliminate malaria should ideally target both the asexual parasites and transmissible gametocytes. Several imidazopyridazines (IMPs) and 2-aminopyridines (2-APs) have been described as potent antimalarial candidates targeting lipid kinases. However, these have not been extensively explored for stage-specific inhibition of gametocytes in *Plasmodium falciparum* parasites. Here, we provide an in-depth evaluation of the gametocytocidal activity of compounds from these chemotypes and identify novel starting points for dual acting antimalarials.

Methods: We evaluated compounds against *P. falciparum* gametocytes using several assay platforms for cross-validation and stringently identified hits that were further profiled for stage-specificity, speed-of-action and *ex vivo* efficacy. Physicochemical feature extraction and chemo-genomic fingerprinting were applied to explore the kinase inhibition susceptibility profile.

Results: We identified 34 compounds with sub-micromolar activity against late stage gametocytes, validated across several assay platforms. Of these, 12 were potent at <100 nM (8 were IMP and 4 were 2-APs) and were also active against early stage gametocytes and asexual parasites, with >1000-fold selectivity towards the parasite over mammalian cells. Frontrunner compounds targeted mature gametocytes within 48 h and blocked transmission to mosquitoes. The resultant chemo-genomic fingerprint of parasites treated with the lead compounds revealed the importance of targeting kinases in asexual parasites and gametocytes.

Conclusion: This study encompasses an in-depth evaluation of the kinase inhibitor space for gametocytocidal activity. Potent lead compounds have enticing dual activities

and highlights the importance of targeting the kinase superfamily in malaria elimination strategies.

INTRODUCTION

Most antimalarial drugs targeting the asexual blood stages of the most lethal malaria parasite, *Plasmodium falciparum*, are not clinically useful against the transmissible, sexual gametocyte forms.[1, 2] Robust *in vitro* gametocyte production and screening platforms[3-5] have allowed evaluation of compound libraries to target *P. falciparum* gametocytes,[6-16] and identify compounds with dual activity against both asexual parasites and gametocytes[17-19], or alternatively, with strategy-specific abilities, targeting either asexual parasites or gametocytes.[18, 19]

An image-based (fluorescence-based confocal imaging of DAPI-stained parasites) high-throughput screen of 36 608 compounds of the BioFocus® DPI SoftFocus® library revealed 222 selective hits against *P. falciparum* parasites.[20] These provided high quality starting points with potent activity against *P. falciparum* asexual blood-stages and physicochemical characteristics representative of “druggable” compounds.[21] Three lead scaffolds, including two kinase focused inhibitor series (2-aminopyridines (2-APs) and imidazopyridazines (IMPs), and an ion-channel focused series of diaminothienyl-pyrimidine (DTP) compounds, were used in hit-to-lead and lead optimisation programs to identify several lead compounds for the IMPs[5, 22, 23], DTPs[22, 24, 25] and 2-APs.[25-28] This includes MMV390048, a phosphatidyl-inositol-4-kinase (PfPI4K) inhibitor currently in clinical trials.[29]

Based on the success of the above-mentioned compounds against asexual blood-stage malaria parasites and proof that at least two of them additionally have gametocytocidal activities,[29, 30] representative compounds from the 2-AP, IMP, and DTP series were further interrogated here for their gametocytocidal activity. Several 2-APs and IMPs were active against both asexual and gametocyte stages and were profiled through sequential and increasingly more demanding chemical and biological filters, to identify potent chemical scaffolds, useful as dual active antimalarial agents.

MATERIALS AND METHODS

Chemistry

Aminopyridine and aminopyrazine (APs) derivatives were prepared from commercially available 2-amino-5-bromopyridine and 2-aminopyrazines, respectively, as described.[26-28, 30] The IMPs were synthesized from 3-amino-6-chloropyridazine.[23, 31] Likewise, the DTPs were synthesized as reported.[22, 24]

In vitro cultivation of asexual stage parasites and induction of gametocytes

All *in vitro* parasite and blood collection work has ethics approvals (University of Pretoria 120821–077; CSIR Ref 10/2011; University of the Witwatersrand M130569). *P. falciparum* asexual cultures were maintained as described before,[32, 33] including the initiation, induction and maintenance of gametocyte cultures from reference strains (drug susceptible NF54).

In vitro P. falciparum antiplasmodial activity and cytotoxicity evaluation

Compounds were screened against *P. falciparum* K1 (chloroquine, mefloquine and pyrimethamine resistant) and NF54 strains *in vitro* using a modified [^{3}H]-hypoxanthine incorporation assay.[34] *In vitro* cytotoxicity was determined by screening compounds against Chinese Hamster Ovarian (CHO) cells, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT),[35] with Emetine as reference. Cells (10^5 cells/mL) were exposed to 10-fold compound serial dilutions for 48 h incubation, in triplicate for two biological replicates. Nonlinear dose-response curve fitting analysis allowed IC_{50} deduction (GraphPad Prism 6, GraphPad Software Inc.).[36]

In vitro gametocytocidal activity evaluation

Gametocytocidal activity was evaluated independently with a gametocyte-luciferase reporter assay (Luc), an ATP assay or a PrestoBlue® assay as previously described.[32] All assays were performed in parallel using the same stock of compounds, diluted fresh with glucose-rich complete culture medium from 1 µM stock solutions in DMSO (final [DMSO] ≤0.5%). Drug controls included methylene blue (25 µM for the ATP assay, 5 µM for the Luc assay) and MMV390048 (5 µM for the Luc assay). Untreated gametocytes and uninfected erythrocytes/culture medium were used to monitor viability and background, respectively. Primary screen data were analyzed using OriginPro 2016 (OriginLab® Corporation) and quality parameters determined by coefficient of variation (%CV), signal-to-background (S/B), signal-to-noise ratios (S/N) and Z'-factor. IC_{50}'s were generated with Graphpad Prism 6, represented as means ± SEM for n≥3 independent biological replicates.

Luciferase Reporter Assay

A luciferase reporter assay for early (NF54-*pfs16*-GFP-Luc) and late (NF54-*mal8p1.16*-GFP-Luc) gametocyte marker cell lines[37] (gift from David Fidock, Columbia University, USA) enabled stage-specific analyses of synchronous gametocyte cultures (2% gametocytaemia, 1.5% haematocrit). Drug assays were performed on early (>90% stage I-III; EG) and late (>95% stage IV/V; LG) gametocytes with 48 h drug pressure as before.[32]

Resazurin-Based Dye Assay

The PrestoBlue® (Life Technologies) assay was performed on semi-synchronous gametocyte cultures (2% gametocytaemia, 5% haematocrit) for 48 h drug pressure on late stage (IV/V; LG) gametocytes.[32]

ATP Bioluminescence Assay

Late stage gametocytes (>95% stage IV/V) were enriched using density gradient centrifugation and magnetic separation as described.[32] Approximately 50 000 gametocytes were exposed to drug for 24 h.

Characterization, cultivation, genotypic and phenotypic profiling of clinical isolates of southern African origin

Isolates were sampled between February - April 2014 and represent a pool of current southern African parasites (ethics clearance University of Pretoria 417/2013, University of the Witwatersrand M140995). *Ex vivo* cultures were initiated from intravenous blood samples within 2-24 h,[33] and parasites only maintained for a maximum 5x *in vitro*

passages. Cryopreserved stocks were prepared throughout to maintain polyclonal variability for all subsequent gametocyte inductions. All the isolates were phenotyped for drug resistance against artemisinin, chloroquine, dihydroartemisinin (DHA), mefloquine, pyrimethamine, amodiaquine using SYBR Green I fluorescence assays and genotyped using PCR and restriction fragment length polymorphism (RFLP) for asexual drug resistance markers *dhfr* (codons 50, 51, 59, 108, 164); *dhps* (codons 436, 437, 540, 581) and *pfcrt* (codon 76) and *pfmdr1* (codons 86). Gametocytogenesis was induced as above and hit compounds evaluated for activity on the resultant late stage (>95% stage IV/V) gametocytes for a panel of 9 gametocyte-producing *ex vivo* African clinical isolates using either the ATP assay (compound evaluated at 1 µM) or with a previously reported pLDH (at 1x IC_{50} of each compound) assay.[38]

Gametocytocidal stage-specificity and speed-of-action

Highly synchronised asexual parasites (>97% rings) were used to induce gametocytogenesis to produce compartmentalised early (>90% stage I-III) and late stage gametocytes (>95% stage IV/V) or mature stage V gametocytes (>95% stage V gametocytes). Full dose-response analysis was performed for each compound after 48 h exposure, after which the luciferase activity was measured as described above. To determine speed of action, early and late stage gametocytes were exposed to compounds for 24, 48 and 72 h at IC_{50} to allow temporal evaluation of IC_{50} fluctuations, (Figure S1). All assays were performed on three independent biological replicates (technical triplicates, GraphPad Prism 6).

Standard membrane feeding assay (SMFA)

SMFA was used to determine the infectiousness of gametocytes as previously described.[39] Mature (≥95% stage V) *P. falciparum* gametocytes were exposed to varying concentrations of MMV642943 (0.001 to 1 μM) for 24 h prior to feeding to *A. stephensi* mosquitoes. Both exflagellation assay and SMFA were performed as previously described.[40, 41]

Physicochemical properties and activity associations

Structure-activity landscape analysis (SALI) and *in silico* predicted partition coefficient (cLogP, atomic cutoff <20) or octanol-water distribution coefficient (logD, pH7.4) was performed using OriginPro 2016 and Osiris DataWarrior V 4.2.2 (www.openmolecules.org). Activity cliff analysis was performed with the SkelSpheres descriptor as similarity criterion and takes into account stereochemistry, duplicate fragments and heteroatom depleted skeletons; similarity threshold at 80%.

DNA microarray analysis

Synchronised asexual NF54 parasite cultures (7-10% parasitaemia, ~90 mL) and late stage gametocyte cultures (2-3% gametocytaemia, stage IV/V, enriched by Nycoprep 1.077 centrifugation) were treated with 10 x IC_{50} of MMV390048 or MMV642943 for 24-48 h. DNA microarray was performed on *P. falciparum* custom Agilent 60-mer 8x15k arrays (AMADID#037237,[42] see supplementary methods). The full dataset is available at the Gene Expression Omnibus (GSE100692).

RESULTS

Hit identification

In total, 66 compounds belonging to the 2-AP (31 representatives), IMP (23 representatives), and DTP (12 representatives) series were evaluated for their activity against late stage gametocytes (>95% stage IV/V). All compounds were cross-validated on three independent assay platforms (Luc reporter, ATP and Prestoblue® assays) to interrogate different biological endpoints and minimize assay interference. Strict selection criteria were imposed requiring primary hits to pass thresholds of >50% (1 µM) and >70% inhibition (5 µM) (Figure 1).[18] Overall, the 2-APs were the most active compounds, with a hit rate of 84% on the Luc assay (Figure 2). By contrast, the DTPs displayed a 33% hit rate on the ATP assay, whereas very similar hit rates of 30-70% were observed for the IMPs, irrespective of the assay platform used. A comparison of the compound efficacy across the three assay platforms indicated high similarity (Pearson r^2=0.82) between the Luc and ATP assays, whereas the PrestoBlue® assay showed a strong correlation for the IMPs with one or the other assay platforms (r^2 of 0.82 and 0.74, respectively).

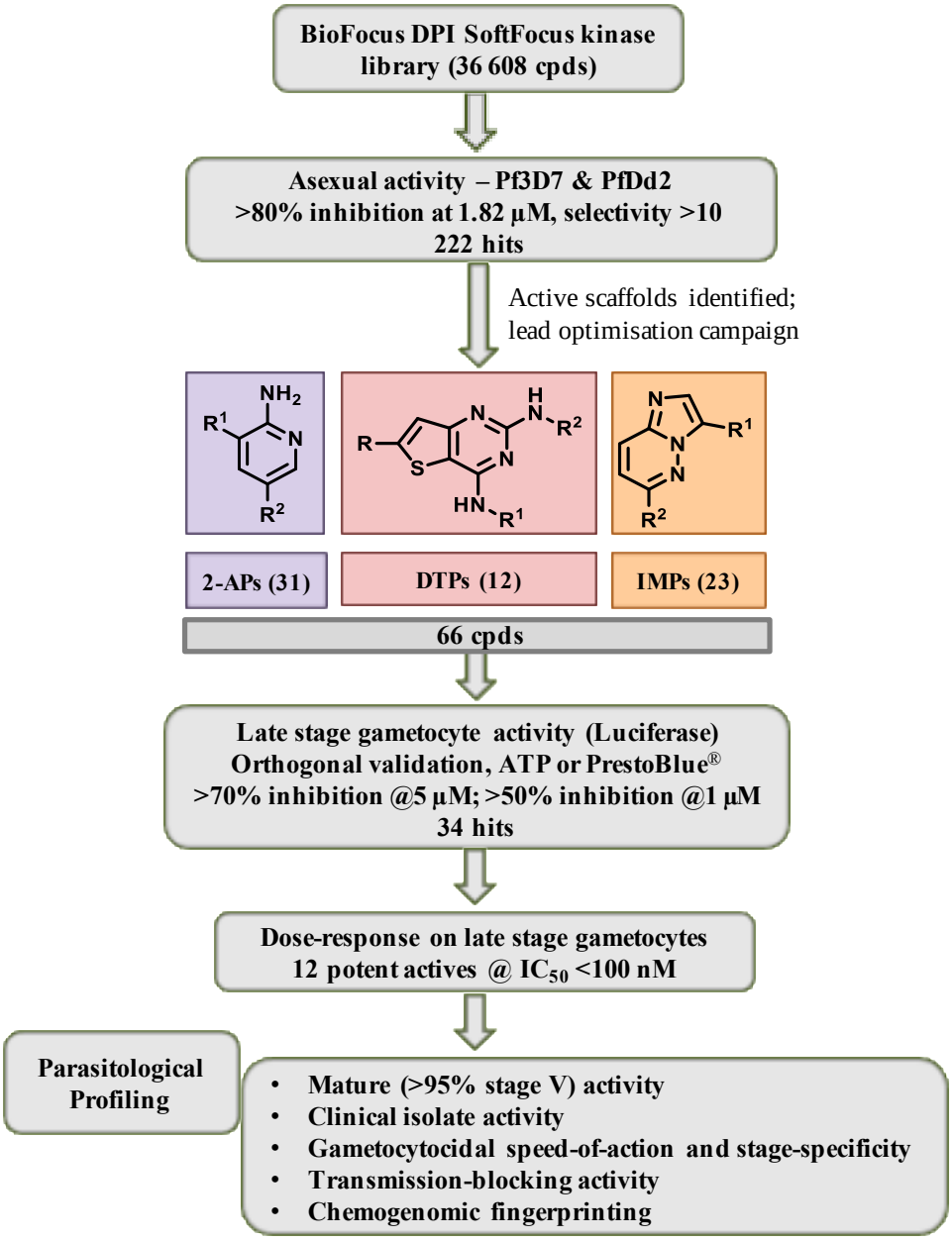


Figure 1: Description of the critical path followed for screening of select scaffolds from kinase libraries for gametocytocidal activity. The DTPs series are indicated in pink, the APs in purple and the IMPs in orange. The number of compounds in each series are indicated in brackets. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

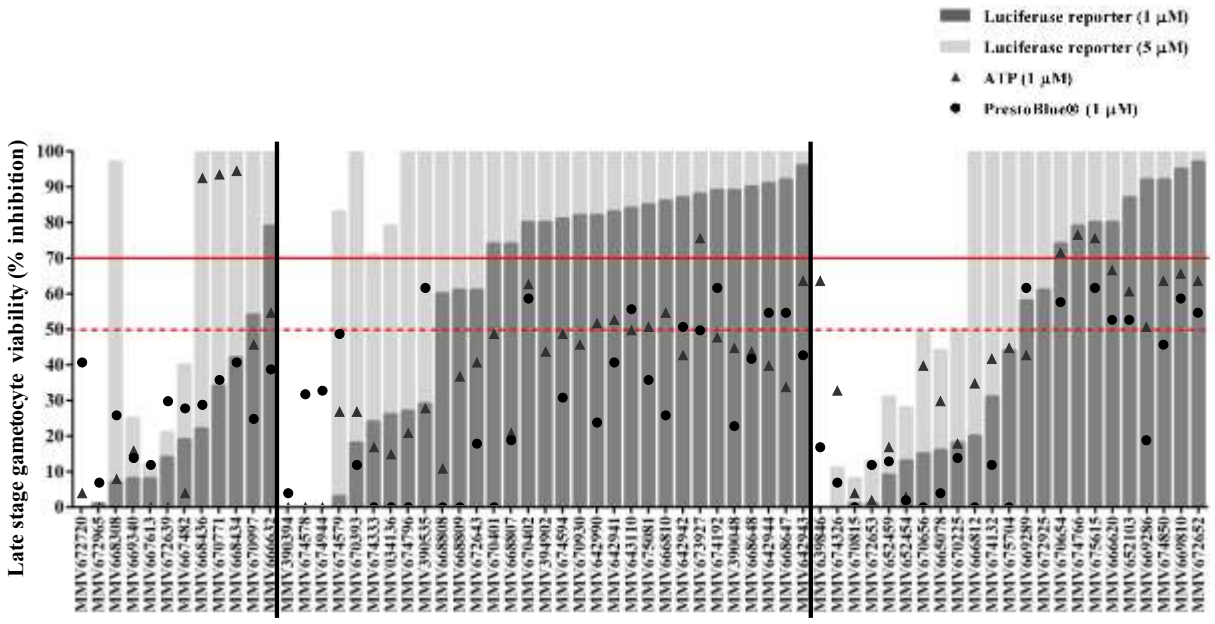


Figure 2: Primary hit identification for late stage gametocytocidal activity. Inhibition of *P. falciparum* mature stage IV/V gametocyte viability after 48 h continuous exposure to 1 and 5 µM drug (DTPs: pink, APs: purple and IMPs: orange) using the luciferase reporter assay (light and dark bars), PrestoBlue® (dots) and ATP (triangles) assay platforms. Data are organised per series and with increasing percentage inhibition on the luciferase platform (1 µM). The 70% (solid red line) and 50% (dotted red line) selection cutoffs are indicated. Results are expressed as percentage inhibition compared to the untreated controls and are representative of single biological replicates, ± SD. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

a

Compound	IC_{50} (nM)	
	Asexual	LG
MMV668434	43	105
MMV666632	28	643
MMV642943	5.7	66
MMV674192	9.2	45
MMV642944	20	52
MMV643110	23	72
MMV642942	10	135
MMV668647	21	137
MMV390048	22	140
MMV673927	15	146
MMV666810	5	179
MMV670930	14	190
MMV394902	19	209
MMV642941	53	225
MMV642990	14	237
MMV670401	42	238
MMV668808	94	342
MMV668809	38	432
MMV670402	26	441
MMV672643	42	460
MMV668648	6.0	536
MMV675081	24	845
MMV668807	26	901
MMV669810	0.5	1.4
MMV669286	0.9	3.0
MMV672652	0.6	3.3
MMV652103	7.3	27
MMV674850	2.7	29
MMV674766	7.9	66
MMV675615	4.5	72
MMV666620	12	79
MMV672925	10	477
MMV670815	417	655
MMV672653	36	907

b

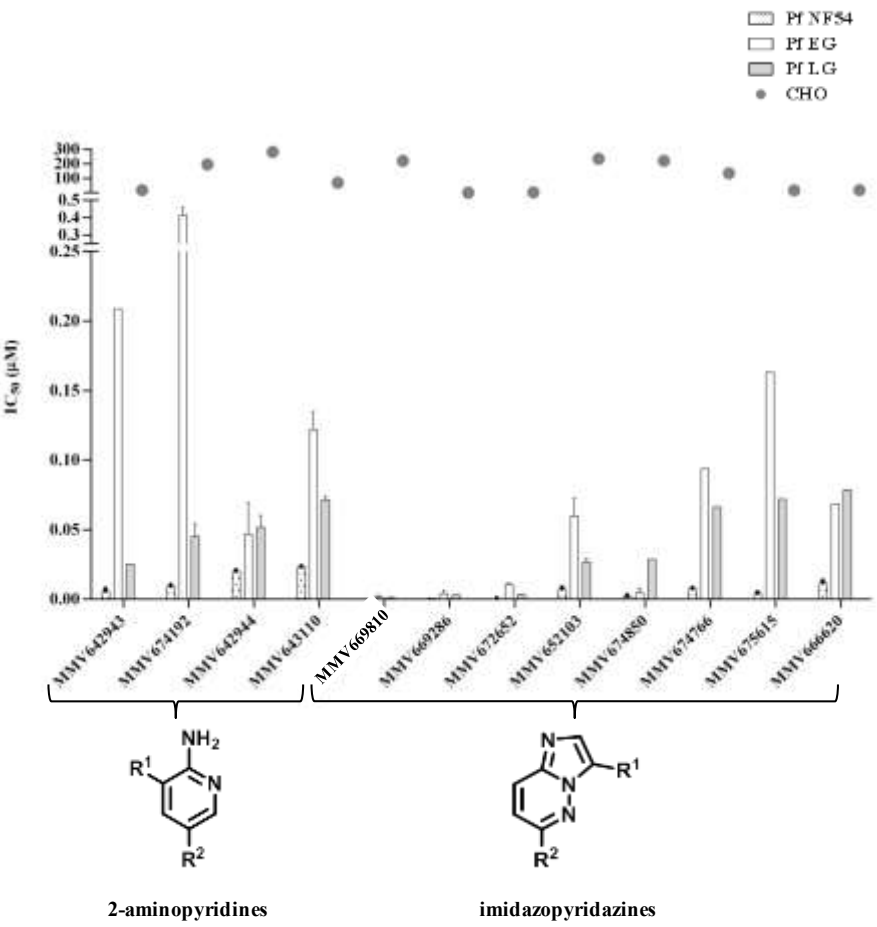


Figure 3: Biological profiles of the evaluated compounds. (a) Stage-specific IC_{50} data are organised per series as increasing IC_{50} values on late stage gametocytes. The DTPs series are indicated in pink, the APs in purple and the IMPs in orange. Colour intensity represents decreasing *in vitro* potency against *P. falciparum* asexual stages, early (stage II/III) gametocytes (EG) and late (stage IV/V) gametocytes (LG). Late stage gametocyte IC_{50}'s represent the lowest of each value obtained for the luciferase reporter (NF54-*mal8p1.16*-GFP-Luc) or ATP bioluminescence assays. **(b)** Biological profiles of hits displaying late stage IC_{50}'s <100 nM are indicated. *P. falciparum* NF54 asexual (patterned), early stage gametocyte (white fill), late stage gametocyte (grey fill) and CHO IC_{50} (dots) values are indicated. Data are representative of at least two biological experiments, each performed in technical triplicates, ± SEM. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

The selected 50 primary hits were evaluated with dose-response to determine their IC_{50} values on late stage gametocytes (>95% stage IV/V), which corresponded well to the inhibition data obtained from the primary screen (Table S1). Of these, 34 compounds had sub-micromolar IC_{50}s (Figure 3a), composed of 6% DTPs, 62% 2-APs and 32% IMPs. These compounds also maintained activity on asexual blood stages (Figure 3a, Table S1), with ~14-fold drop in inhibitory activity between asexual parasites and gametocytes. However, the rank order of compounds with activity against asexual parasites and active against gametocytes differed, indicating that preference towards specific gametocyte stages is present even within clusters of compounds with dual gametocyte-asexual activity (Table S1).

The 12 most potent compounds (Figure 3b) on late stage gametocytes include 8 IMPs (MMV669810 IC_{50} = 1.4 nM; MMV669286 = 3 nM; MMV672652 = 3.3 nM; MMV652103 = 27 nM; MMV674850 = 28.7 nM; MMV674766 = 66.4 nM; MMV675615 = 72.1 nM; MMV666620 = 78.9 nM) and 4 2-APs (MMV642943 IC_{50} = 66 nM; MMV674192 = 45.2 nM; MMV642944 = 51.9 nM; MMV643110 = 71.5 nM) (Table S1). All of these compounds have dual activity between asexual parasites and gametocytes and displayed minimal cross-resistance to the *P. falciparum* multidrug resistant K1 strain, with resistance indices <10 (Table S1). Additionally, the majority of the compounds were highly selective towards *P. falciparum* asexual parasites (>1000-fold) and late stage gametocytes (>10-fold) compared to mammalian cells (Figure 3b, Table S1).

Hit profiling

The activity of selected gametocytocidal compounds was evaluated against late stage gametocytes from currently circulating clinical isolates of southern African origin (Figure

4). These clinical isolates were genotyped and phenotyped for selected drug resistance markers on asexual parasites (Table S2).[43] The majority of 2-APs and IMPs (but not the DTPs) were comparably active against gametocytes from the clinical isolates and the *P. falciparum* NF54 reference strain, with the highest activity maintained even in isolates with resistant phenotypes (e.g. JZA 20 and TD_01, both antifolate resistant). MMV642944 and MMV642942 were consistently more active against the clinical isolates compared to the NF54 reference strain. Collectively, this indicates activity towards contemporary clinical isolates of geographical relevance.

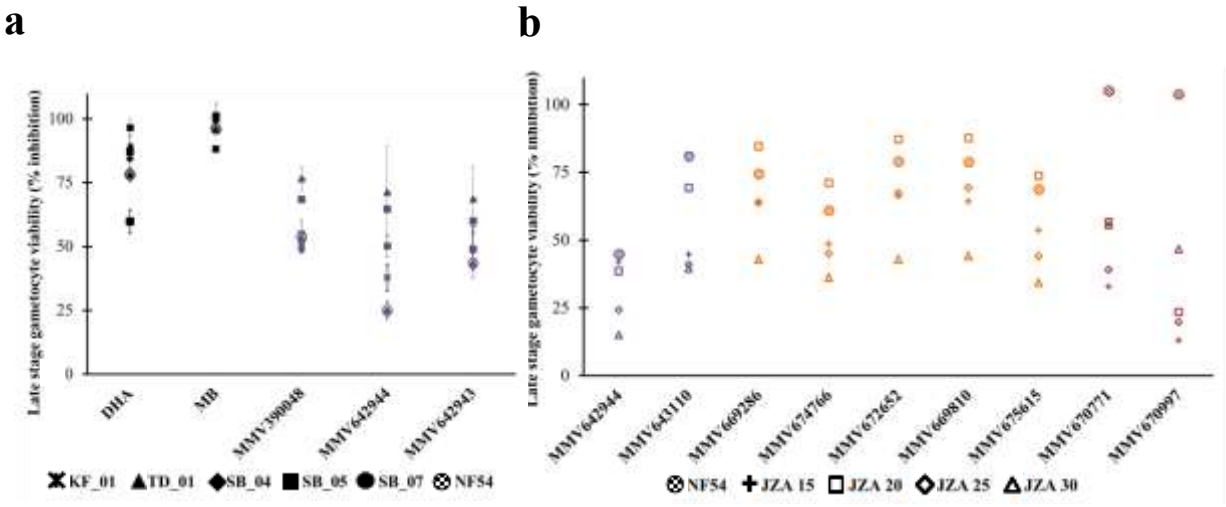
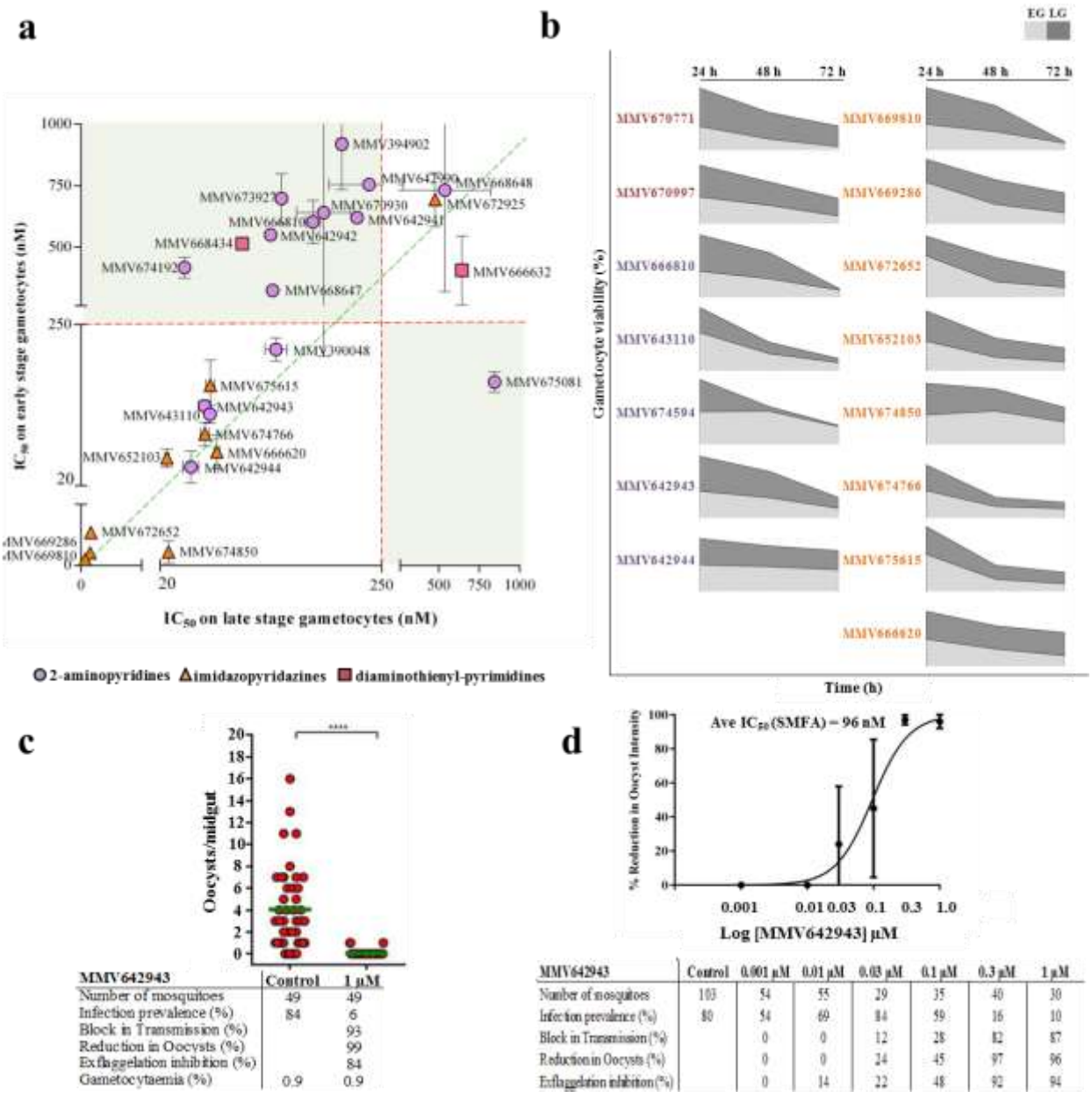


Figure 4: Late stage gametocytocidal activity of the lead compounds against *ex vivo P. falciparum* clinical isolates. **(a)** The pLDH assay was performed at 1 x IC_{50} for each compound, as previously determined on the NF54-*mal8p1.16*-GFP-Luc strain using the luciferase reporter assay. **(b)** The ATP assay was performed at a single concentration of 1 µM. In all instances, *P. falciparum* NF54 was included as drug susceptible reference strain and MB (5 µM) and DHA (100 µM) as reference drug controls. Compounds are coloured according to classes as in Table S2. Data are representative of at least triplicate experiments ± SEM; a single biological repeat was performed for SB_07. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.



MMV642943	Control	1 µM
Number of mosquitoes	49	49
Infection prevalence (%)	84	6
Block in Transmission (%)		93
Reduction in Oocysts (%)		99
Exflaggelation inhibition (%)		84
Gametocytaemia (%)	0.9	0.9

MMV642943	Control	0.001 µM	0.01 µM	0.03 µM	0.1 µM	0.3 µM	1 µM
Number of mosquitoes	103	54	55	29	35	40	30
Infection prevalence (%)	80	54	69	84	59	16	10
Block in Transmission (%)		0	0	12	28	82	87
Reduction in Oocysts (%)		0	0	24	45	97	96
Exflaggelation inhibition (%)		0	14	22	48	92	94

Figure 5: Dual reactivity of the lead compound series toward *P. falciparum* different gametocytes developmental stages. **(a)** Early (>95% II/III; y-axis) and late stage (>95% IV/V; x-axis) gametocytes were assayed after 48 h continuous exposure to drug using the luciferase reporter and/or ATP bioluminescence assays and the IC_{50} determined using GraphPad Prism. Dual-active compounds are situated on the trend line (dotted, green). Fold changes for stage-specificity towards either early or late stage gametocytes are indicated. **(b)** Speed of action for selected compounds, with gametocyte viability determined for 24, 48, or 72 h drug pressure at 1 x IC_{50} (determined at 48h). Dark grey = late stage

gametocytes, light grey = early stage gametocytes. Data are representative of at least three biological experiments, each performed in technical triplicates, ± SEM. **(c)** Effect of MMV642943 on oocyst intensity after 24 h treatment (1 μM). The Mann Whitney Test was used to compare the statistical significance between the drug treatments and control. The total number of oocysts in a single mosquito midgut (red dots) and mean oocyst intensity of infection (green line) are indicated. *P*<0.0001 as ****. **(d)** Dose-response of MMV642943 effect on oocyst intensity. Data are representative of at least three biological experiments, each performed in technical triplicates, ± SEM, for a minimum of 17 fed mosquitoes at highest concentration tested. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

The parasitological properties of the 34 compounds with late stage IC_{50}s <1 μM (from Figure 5a) were additionally evaluated on both early (>90% stage I-III) and late (>95% stage IV/V) stage gametocytes (Figure S1 and S2). Within the 2-APs, the majority (88%) was significantly more active (*P*<0.005, n=3) towards late stage gametocytes (Figure 5a, Table S1) as was true for the two drug candidates MMV390048 (EG IC_{50} = 214.6 nM; LG IC_{50} = 140.3 nM) and MMV642943 (EG IC_{50}= 134 nM; LG IC_{50} = 66 nM).[29, 30] Within the IMPs, the most active compounds showed comparable activity (<2-fold difference) for both early and late stage gametocytes (MMV669810 and MMV669286).

The stage-specific gametocytocidal activity of selected hits from all three series was confirmed via additional evaluation of their speed-of-action over 24, 48 or 72 h (Figure 5b) using each compound at 1 x IC_{50} (determined at 48 h). Treatment for shorter periods (<12 h) did not result in accurate dose-responses for any compound. Within the 2-APs, MMV642943 and MMV642944 had similar slow speed of action

against both early and late stage gametocytes (>24 h lag). By contrast, MMV674594 required 48 h to affect early stage gametocytes, but 72 h to affect late stage gametocytes. Within the IMPs, several of the hits had a similar speed of action with a lag of 48 h on both early and late stage gametocytes (e.g. MMV669286, MMV672652 and MMV652103). MMV669810 preferentially targets early stage gametocytes within 24 h. The DTP compounds kill both early and late stage gametocytes slowly.

To interrogate late stage gametocyte activity, a subset of compounds was further evaluated against >95% pure stage V, mature gametocytes (Table S1). The majority of 2-APs had <2-fold difference in activity between mixed stage IV/V and pure stage V, including clinical candidate MMV390048 and pre-clinical candidate MMV642943. This ability translated directly into blocking transmission to mosquitoes, as evident from a significant reduction in oocyst density for both MMV390048[21] as well as MMV642943 (99% reduction in oocysts, Figure 5c). MMV642943 resulted in 93% block in transmission with an IC_{50} of 96 nM in SMFA (Figure 5d) compared to an IC_{50} of 111 nM for MMV390048.[21] The IMPs also displayed equipotent activity against stage IV and V gametocytes with MV669810 at 1.4 nM versus 1.2 nM, respectively. This confirms that these compounds are able to target and kill mature, transmissible gametocytes.

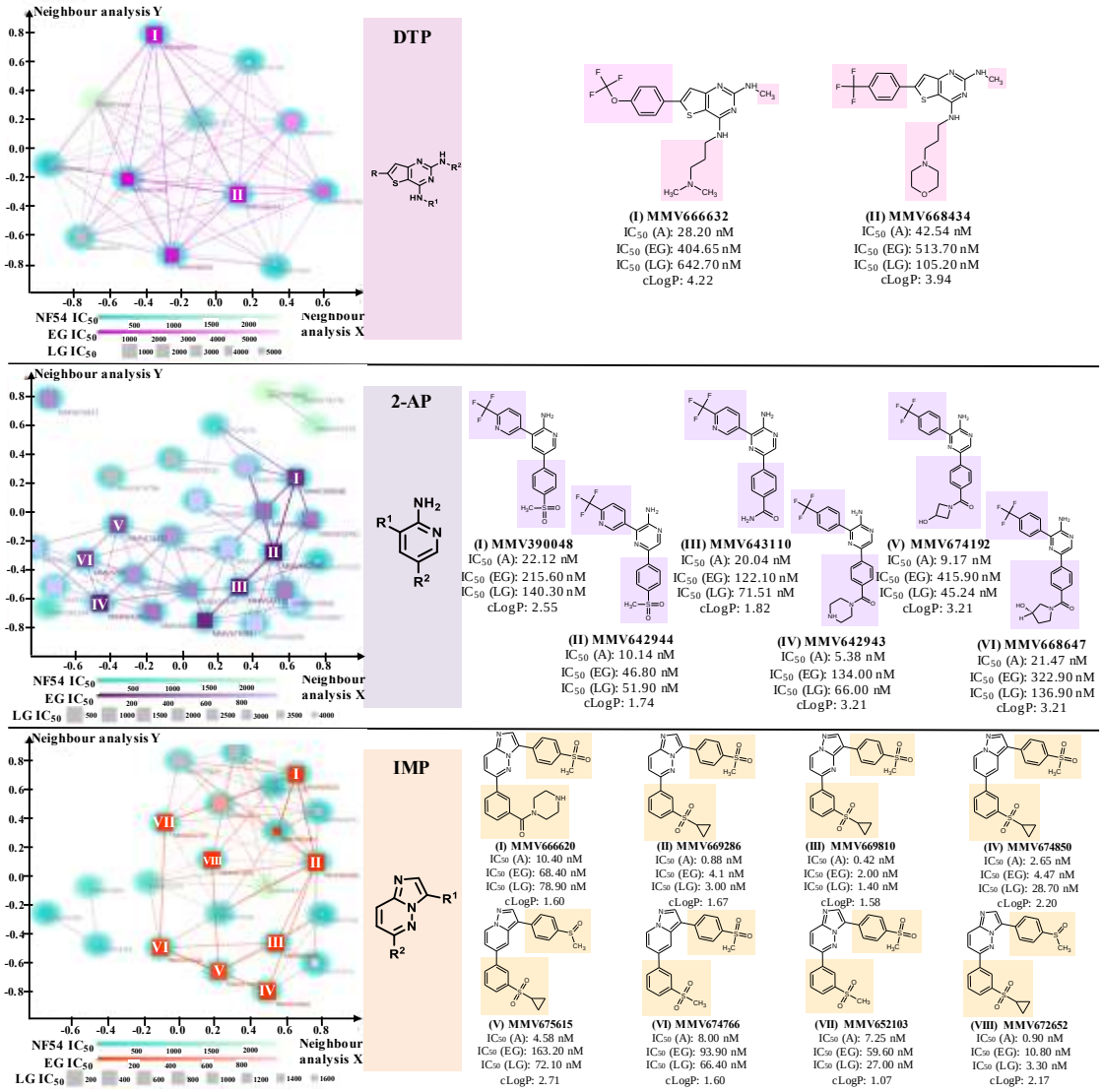


Figure 6: Intra-series structure activity landscape interrogation of the three lead series. Pairwise late (IV/V) stage gametocyte activity to structural feature (SkelSphere) analysis was performed with superimposed activity cliff analysis (Osiris DataWarrior V 4.2.2) separately on each series at a stringency of 80% in structural characteristics. The DTP series are indicated in pink, the 2-APs in purple and the IMPs in orange. The same scheme was applied to the R-groups. IC_{50} inclusions are limited to those compounds with sub-micromolar activity; A = asexual parasites; EG = early stage gametocytes; LG = late stage gametocytes. Data are representative of at least triplicate biological experiments. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Physicochemical space extraction for dual activity

Subsequent preliminary structure-activity relationships (SAR) between and within the three series were inferred by performing chemical feature extraction and biological activity association. Intra-series delineation of the hit compounds was subsequently performed (Figure 6), with very little inter-series overlap observed (Figure S3a). Within the DTP series, the thienylpyrimidine backbone was an active representative. The methylation at position R^2 and the phenyl fragment at R^1, which were previously shown to be required for potent *in vitro* asexual activity,[22] were confirmed here to be important for the gametocytocidal activity of this chemotype, which was enhanced with lipophilic groups at the phenyl ring *para*-position.

Dual active compounds within the 2-AP series polarized into two clusters based on chemical similarity. The first cluster contained 3,5-diaryl-2-APs MMV390048 (and its 2-aminopyrazine derivatives, MMV642944 and MMV643110) and the hits MMV642942 and MMV675081, with both asexual[21] and gametocytocidal activity. The 2-aminopyrazine backbone was identified during SAR explorations around the pyridine core and led to improvements in late stage gametocyte activity (average pyridine IC_{50} 140 nM; average pyrazine IC_{50} 49 nM). Separated from the first cluster was a second cluster (MMV642943, MMV674192 and MMV668647), containing R^1 trifluoromethylphenyl substitutions with various amide moieties at R^2. These compounds had lower predictive hydrophilicities (cLogP >3) with associated >10-fold activity loss against early stage gametocytes.

Within the IMPs, a homogeneous association was observed between compound activities and chemical features. Similar to the 2-APs, *para*- and *meta*-phenyl

substitutions at positions 3 and 6, respectively, of the imidazopyridazine core were essential for *in vitro* asexual[23] and gametocytocidal activity. The R^2 cyclopropylsulfone and R^1 methylsulfonyl improved overall activity of the series. The nitrogen at position 5 of the imidazopyridazine core further enhanced the activity of these compounds to <10 nM and was particularly important for late stage gametocyte activity, but dependent on the R^2 cyclopropylsulfone.

Functional evaluation of dual active kinase inhibitors

To understand the functional consequence of inhibiting lipid kinases in the parasite, we used global gene expression analysis to evaluate the response of both asexual parasites and gametocytes following inhibition with the *Pf*PI4K inhibitors MMV390048 and MMV642943.

An appreciable and tractable drug-specific effect was observed on asexual parasite transcriptomes when treated for 48 h, with 15-29% of the transcriptome differentially affected (log_2 fold-change thresholds at 0.5 and -0.5, Supplementary file 1). However, a detectable transcriptional response was not observed at 24 h, which confirms the described moderately slow action of these compounds, with a 24 h lag period.[29] MMV390048 and MMV642943 treated asexual parasites responded nearly identically (Pearson correlation coefficient of 0.96, Figure 7a). The resultant transcriptional profiles were unique and did not show close association with other antimalarials (Figure 7b).[44] An enrichment map of the biological processes affected due to MMV390048 or MMV642943 treatment using moderately stringent statistical significance (P <0.05, FDR <0.1%) included 47 gene sets overrepresented after treatment (Figure 7c). A variety of different processes were affected as expected after

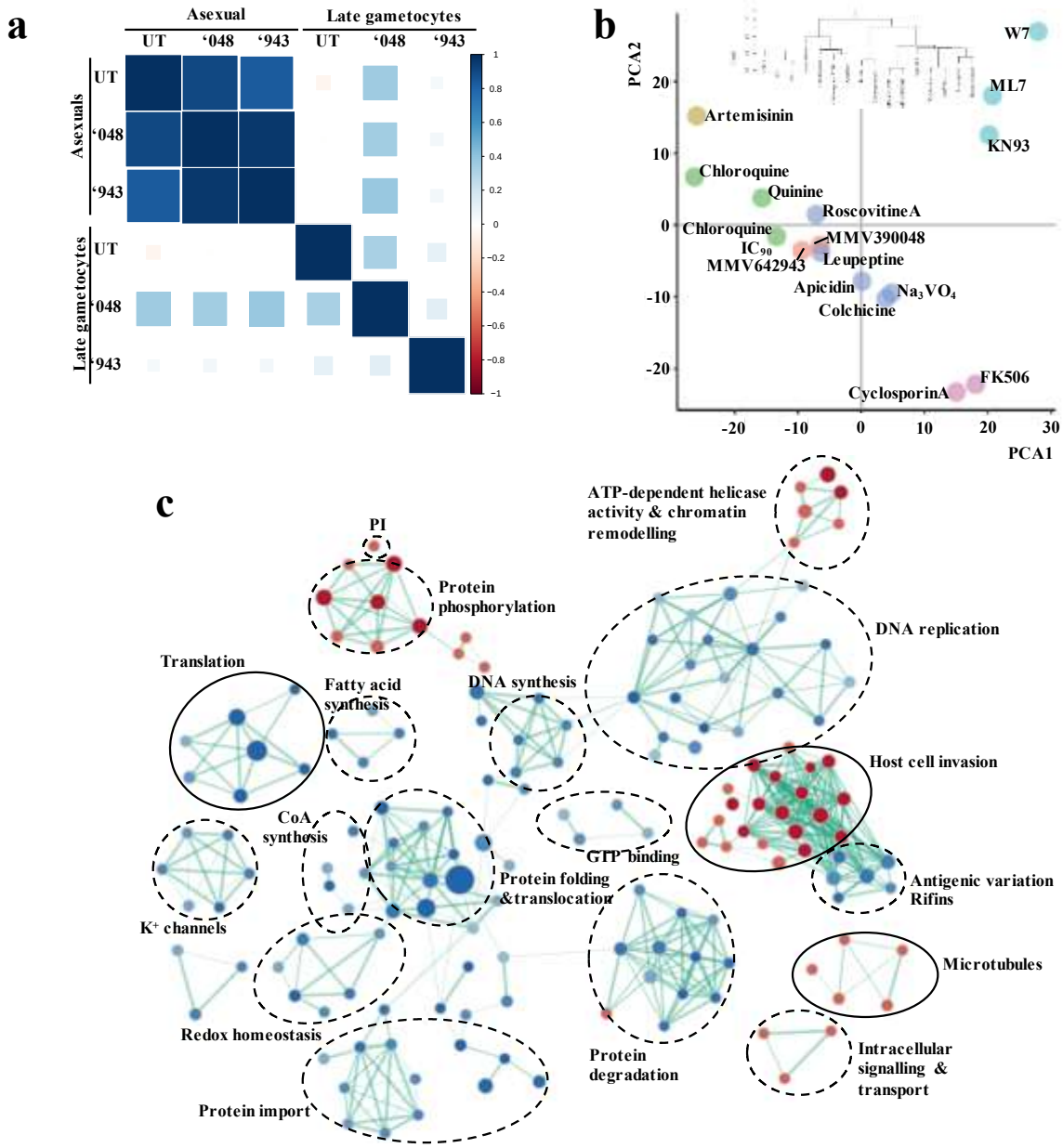


Figure 7: Transcriptional response evaluation of parasites treated with kinase inhibitors. (a) Pearson correlation analysis of the complete transcriptional profiles of either asexual parasites or late stage gametocytes treated with compound MMV390048 (0048) or MMV642943 (943). **(b)** PCA analysis of MMV390048 and MMV642943 treated asexual parasites compared to the transcriptome of parasites with numerous other perturbations as described.[44] **(c)** Gene set enrichment map for *P. falciparum* parasites treated with MMV390048 and MMV642943. GSEA was performed with $P<0.05$ and FDR<1% using hierarchical clustering within nodes. Node sizes represent the number of members present in the

node. Processes enriched in the treated population are indicated in red, whilst blue indicates processes enriched in normal parasites. Hashed circles are processes only observed in asexual parasites, solid circles are processes observed in both asexual parasites and late stage gametocytes**.** This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

inhibition of proteins involved in phosphatidyl-inositol 4-phosphate (PI4P) synthesis, which has pleiotropic functions involved in intracellular signalling and membrane maintenance. Protein phosphorylation, host cell invasion and intracellular signalling/transport were three particularly strongly affected communities, with multiple members within the nodes showing decreased gene expression after treatment, except for intracellular transport processes that showed increased abundance after treatment (Figure 7c). Moreover, processes like macromolecular synthesis, protein degradation and protein folding and translocation were severely affected in the treated populations, linked to *Pf*PI4K inhibition. The transcriptional fingerprint for late stage gametocytes (Supplementary file 1) displayed similarly affected different biological processes including decreased macromolecular synthesis, lipid synthesis, gene expression and host cell invasion. However, life cycle differences between asexual parasites and gametocytes were evident, as a reduction in protein degradation observed in asexual parasites was not enriched in gametocytes, which do not digest haemoglobin. This implies some divergence in the effect of inhibiting *Pf*PI4K in different life cycle stages.

DISCUSSION

Dual active compounds are primary starting points for the generation of antimalarials that are able to treat clinical symptoms and are useful to eliminate malaria.[18] To our knowledge, this study provides the most in-depth evaluation of the lipid kinase inhibitor space for gametocytocidal activity and identifies compounds useful as starting points for development of dual active antimalarials. Additionally, the potent activities observed against late stage gametocytes entice strategies prioritising gametocyte-only activities as part of the newly defined target candidate profiles.[19, 45]

Our data indicate that, although some potential ion-channel modulators show potency against asexual blood stage parasites and gametocytes (e.g. *Pf*ATP4 inhibitors),[14, 46] those tested here are generally poor inhibitors of gametocytogenesis. By contrast, compounds selected for their kinase inhibitor chemical background yielded very active gametocytocidal compounds, whilst additionally maintaining their asexual activity. A 2- to 10-fold higher hit identification rate for the compounds tested here, compared to those typically seen by screening diversity oriented libraries[9, 11, 14, 15, 39, 47, 48] supports the chemical signature for kinase inhibition previously alluded to as gametocytocidal.[14, 41] A total of 34 compounds were identified with sub-micromolar activity against late stage gametocytes, of which 12 were potent at <100 nM. Cross-validation of these hits on independent assay platforms and the presence of high selectivity towards the parasite, provide confidence to the potency and usefulness of these hit compounds.

Gametocyte stage-specificity and speed-of-action of such potential transmission-blocking actives importantly confirmed 87% of the hits (<100 nM activity) identified on

stage IV/V populations maintained their inhibition against mature stage V gametocytes. The majority of the compounds acted after a 24 h lag period on various stages of gametocytes, similarly to the clinical candidate MMV390048[29] and this effective timeframe is sufficient to result in transmission-blocking activity evident for the lead drug candidates (MMV390048[29] and MMV642943 reported here). The luciferase assay on late stage gametocytes therefore has some predictive nature for transmission-blocking activity, confirming previous reports.[49] A subset of compounds preferentially targeted late stage gametocytes, similarly to what has been observed during other screens,[14] raising questions as to the enhanced permeability of late stage gametocytes with a ghost-like erythrocyte and expression of perforin-like proteins.[50] These compounds were characterized as having sulfone/sulfoxide moieties with associated potential membrane permeability limitations in the 2-AP series, but not for the IMPs. This implies that predictive hydrophilicity is not a general indicator of differential activity of compounds against different gametocyte stages. The different stage-specific preferences between compounds within the IMPs and 2-APs may indicate a propensity to target multiple members of structurally related parasite kinases whilst maintaining sufficient selectivity from human kinase inhibition.[16] This scenario may indeed be preferred to inhibitors targeting single kinases that may be more prone to resistance development[51] as has been observed for imidazopyridazines that target multiple kinases depending on the different parasite stages during asexual schizogony (e.g Ca^{2+}-dependent protein kinase 1[52] and protein kinase G[53]). This late stage gametocyte preference provides appealing starting points from a transmission-blocking perspective for TCP-5 strategies.[19] Indeed, development of these as gametocyte only compounds

would require new pharmacokinetic (PK) models, independent of constraints that would be associated with PK modelling of dual active antimalarials.

Several kinases that are associated with gametocytes/gametes[51] are clearly druggable,[54] including lipid kinases like *Pf*PI3K and *Pf*PI4K, particularly in late stage gametocytes.[14, 15, 55] Chemogenomic fingerprinting of MMV390048 and MMV642943 revealed clearly differentiated global effects between asexual parasites and gametocytes, as proteolysis required for haemoglobin digestion in asexual parasites is not observed as affected in gametocytes. Moreover, shared processes required for cytokinesis[55] are observed resulting in dysregulation of invasion processes during schizogony and in late stage gametocytes in preparation for egress during gametogenesis. Although the transcriptome of gametocytes differs from that of asexual parasites,[56] the altered intracellular distribution of PI4P after *Pf*PI4K inhibition[55] does affect processes in gametocytes (e.g. DNA replication, cytokinesis and chromatin condensation for gamete formation) that rely on PI signalling and lipid transport. This is driven by phospholipase C (PLC)-mediated[57] production of inositol triphosphate (IP3) from phosphatidylinositol-4,5-bisphosphate (PIP2)[58], which can result in Ca^{2+}-mediated activation of CDPK4 and Map-2[59], that drives processes such as cytokinesis.[51, 60] *Pf*PI4K thus initiates a signalling hub involved in a number of core parasite processes during various stages of development.

The potent activity of the 2-APs and IMPs against gametocytes expands the susceptibility profile of these chemotypes, potentially targeting lipid kinases in various stages of malaria parasites. The hit compounds reported here are enticing starting

points for the development of dual active antimalarial compounds or targeting only gametocytes in transmission-blocking strategies.

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Transparency declaration

None to declare

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Supporting Information.

Supplementary methods, Table S1, Table S2 and Figures S1, S2 and S3 are available online.