Exploring the transmission-blocking activity of antiplasmodial 3,6-diarylated imidazopyridazines

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Abstract

The effectiveness of current antimalarial therapies that cure patients of the pathogenic asexual blood stages is rapidly declining due to the spread of antimalarial drug resistance. This requires the development of novel chemotypes curative for asexual blood stages but additionally, such chemotypes should also target the sexually differentiated gametocytes and thereby block disease transmission. Kinase inhibitors, specifically imidazopyridazines, were previously described as highly effective, dual-active compounds *in vitro*. However, amongst other shortcomings, poor solubility and cardiotoxicity risks prevented these compounds from being further developed. In a recent study, novel 3,6-diarylated imidazopyridazine derivatives showed improved solubility and a decrease in inhibition of the human *ether-a-go-go*-related gene (hERG), suggesting reduced cardiotoxicity risks, with potent sub-micromolar antiplasmodial activities. Here, we report the *in vitro* activity of these 3,6-diarylated imidazopyridazine derivates against both asexual blood and gametocyte stages of the human malaria parasite, *Plasmodium falciparum, in vitro*. We highlight several potentially dual-active compounds with nanomolar activities (IC_{50}'s 0.7–104 nM) against both drug sensitive and resistant strains of *P. falciparum* with these compounds also displaying activity against transmissible gametocytes (IC_{50}'s 1180.3–1787.5 nM). Taken together, the new generation 3,6-diarylated imidazopyridazines have potent activity against *P. falciparum* parasites *in vitro* with improved physicochemical and toxicity profiles.

Keywords: malaria, kinase inhibitors, imidazopyridazines, antiplasmodial, antimalarial, transmission-blocking

INTRODUCTION

Malaria is caused by *Plasmodium* parasites of which *Plasmodium falciparum* is responsible for the highest disease incidence and mortality in sub-Saharan Africa. Despite a steady decline in disease incidence over the past decade, effective malaria elimination strategies are continuously threatened by the rapid spread of antimalarial drug resistance (WHO, 2019). *P. falciparum* parasites are transmitted by female *Anopheles* mosquitos and have a complex, multi-stage developmental programme that mediates the development of pathology and continued disease transmission. The proliferative asexual blood stages (undergoing schizogony every 48 h and marked by development from ring to trophozoite and schizont stages) are responsible for disease symptoms, and the sexually differentiated gametocyte stages are transmitted to *Anopheles* mosquitoes following a blood meal. Gametocytogenesis is a uniquely prolonged process in *P. falciparum* (10–14 days) where five distinct stages can be identified during development and differentiation (stages I-V).

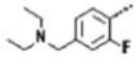
Current antimalarials mainly target the asexual blood stages, thereby alleviating disease symptoms and preventing death. However, drug discovery efforts now also aim to describe additional life cycle activity for newly discovered molecules, including the ability to circumvent disease transmission by targeting the sexual, transmissible gametocyte stages (Birkholtz *et al.*, 2016). Such dual-active compounds will therefore be effective in curing pathology whilst simultaneously contributing to disease elimination.

Protein kinases are second only to G-protein coupled receptors in the number of screening targets that are used by the pharmaceutical industry to identify new therapeutic chemical entities (Zhang *et al.*, 2009), with 25–30% of these targets representing kinases (Kini *et al.*, 2017). Indeed, the kinome and phosphatome constitute ~2% of the *Plasmodium* genome and are thought to play significant roles during life cycle transitions in the parasite due to their involvement in several essential signalling pathways (Anamika *et al.*, 2005, Ward *et al.*, 2004). *Plasmodium* spp. contain between 85 and 99 kinase-associated transcripts, producing 65 confirmed eukaryotic protein kinases (Ward *et al.*, 2004, Anamika *et al.*, 2005). Kinase inhibitors are indeed well described as antimalarial compounds (Lemercier *et al.*, 2009, Brunschwig *et al.*, 2018, Paquet *et al.*, 2017, Singh *et al.*, 2017, Le Manach *et al.*, 2015b, González Cabrera *et al.*, 2018, van der Watt *et al.*, 2018) and have resulted in the delivery of compounds that target *P. falciparum* phosphatidylinositol-4-kinase (PI4 K) as pre-clinical (UCT943) (Brunschwig *et al.*, 2018) and clinical (MMV048) (Paquet *et al.*, 2017) candidates.

The kinase inhibitors under investigation here include derivatives around the imidazopyridazine scaffold, which has previously been ascribed with antiplasmodial activity (McNamara *et al.*, 2013, Sahu *et al.*, 2011, S. Osborne *et al.*, 2011, Lemercier *et al.*, 2009) but also displays anticancer potential (Matsumoto *et al.*, 2013) and has potential in the treatment of anxiety (Moran *et al.*, 1986). An investigative high-throughput screen (HTS) of the BioFocus DPI SoftFocus kinase library (Duffy and Avery, 2012) identified several imidazopyridazine hit compounds with sub-micromolar activity and > 10-fold selectivity (Le Manach *et al.*, 2014a) against both drug sensitive and resistant forms of asexual blood stage *P. falciparum*. Hit-to-lead medicinal chemistry programmes led to a lead compound (MMV652103, Table 1) with di-methylsulfonylbenzene substitutions in both 3 and 6 positions, that was potently active against *P. falciparum* at 7.3 nM with good oral efficacy (98% at 4 × 50 mg/kg) in a *P. berghei* mouse model (Le Manach *et al.*, 2014a, Le Manach *et al.*, 2015b, Le Manach *et al.*, 2014b). Moreover, MMV652103 and derivatives displayed potent activity against sexual stage *P. falciparum* gametocytes [e.g. 27 nM, (van der Watt *et*

al., 2018)], validating their efficacy against multiple life cycle stages and their potential to be used in transmission-blocking strategies.

Table 1. Precursor and 3,6-diarylated imidazopyridazine substituents with *in vitro* asexual blood (ABS) and late gametocyte (LG) stage activities as outlined by SALI-analysis.

Compound name	6-group	3-group	IC_{50} ASB (nM)	IC_{50} LG (nM)	Solubility (μM, pH 6.5)[c]	IC_{50} hERG (μM)[d]
MMV652103[a]			27	<5[b]	7	3.61 ± 0.623
MMV669289[a]			96	1489	<5	ND
MMV670654[a]			35	7993		
MMV672653[a]			35	907.3	186	ND
1			104.0 ± 8.7	5141	160	ND
3			524.1 ± 10.2	4838		
4			ND	>5000		
6			ND	>5000	165	ND
7			ND	>5000	150	ND
8			ND	2135	60	ND
9			261.0 ± 30.1	4231.0 ± 1647.8	<5	ND
10			77.7 ± 4.2	1180.3 ± 117.8	<5	ND
11			ND	>5000	200	ND
12			ND	5357	5	ND
13			ND	>5000		
14			700	>5000		

Compound name	6-group	3-group	IC_{50} ASB (nM)	IC_{50} LG (nM)	Solubility (μM, pH 6.5)[c]	IC_{50} hERG (μM)[d]
15			0.7 ± 0.6	1787.5 ± 699.3	200	0.59 ± 0.02
16			924.8 ± 196.3	7255	200	7.83 ± 1.21

[a]Data for precursor imidazopyridazines (MMV669289, MMV652103 and MMV672653) from (van der Watt et al., 2018).
[b]Solubility data for precursor imidazopyridazine (MMV652103) from (Le Manach et al., 2014b).
[c]Solubility determined using a kinetic (turbidimetric) solubility assay (Cheuka et al., 2018).
[d]hERG inhibition data was determined using the QPatch Clamp System (Cheuka et al. 2018).
Where shown IC50 values are means from three independent biological repeats performed in technical triplicates, ± S.E.

However, poor solubility coupled with cardiotoxicity risks have prevented these compounds from further progression (Le Manach *et al.*, 2015a, Le Manach *et al.*, 2014a). Therefore, strategies to improve address these liabilities were devised and implemented (Figure 1) (Cheuka *et al.*, 2018). The derivatives generated maintained their sub-micromolar activities against *P. falciparum* parasites and displayed good selectivity against mammalian CHO cells. Importantly, some compounds had improved solubility and cardiotoxicity risks as evidenced by reduced hERG inhibition (Cheuka *et al.*, 2018). However, the ability of these new derivatives to target the sexual gametocyte stages and thereby prevent transmission is unknown.

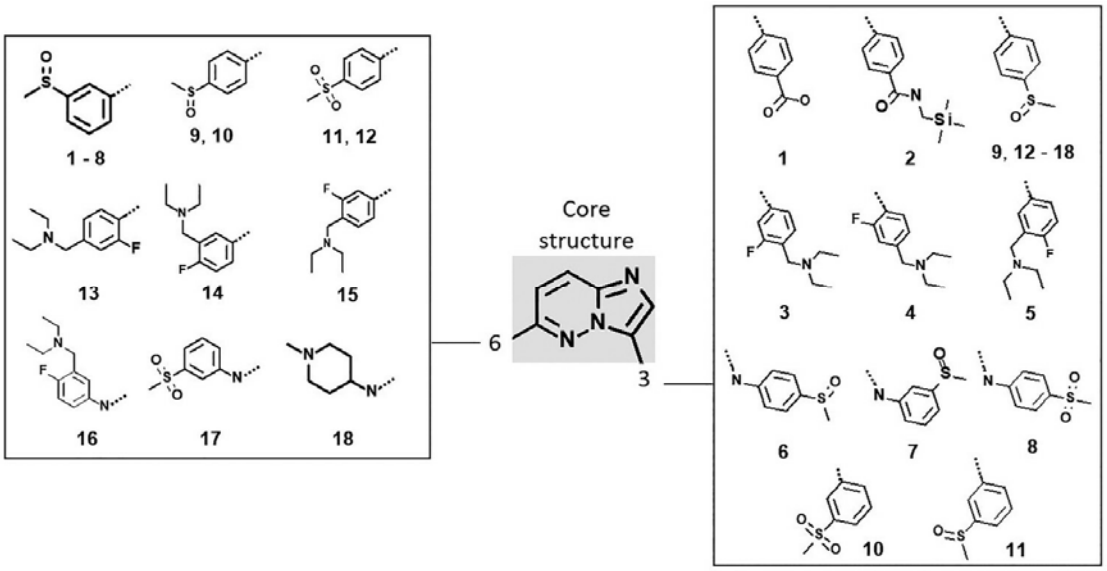


Figure 1. 3,6-diarylated imidazopyridazine analogues. Substitutions at positions 3 and 6 of core imidazopyridazine scaffold.

Here, we performed a parallel evaluation of the *in vitro* activities of 3,6-diarylated imidazopyridazines (Figure 1) (Cheuka *et al.*, 2018) against asexual blood stage *P. falciparum* parasites as well as early- and late-stage gametocytes and male gametes. Through chemical and activity profiling, we highlight potential dual-active 3,6-diarylated imidazopyridazines that can serve as starting points to develop novel therapies that selectively target kinases and thereby aid in malaria elimination strategies.

MATERIALS AND METHODS

Compounds

Compounds 1–18 were obtained from our in-house chemical library. The syntheses and characterisation of the compounds have been previously described (Cheuka *et al.*, 2018).

Ethics statement

This work holds ethics approval from the University of Pretoria Health Sciences Ethics Committee (506/2018).

In vitro culturing of *P. falciparum* asexual parasites and gametocytes

Asexual *P. falciparum Pf*NF54 (drug sensitive) and *Pf*K1 (resistant to chloroquine, pyrimethamine and sulfadoxine) strains were obtained from the Malaria Research and Reference Reagent Resource Center (MR4 BEI resources, Manassas, USA). Asexual blood stage parasites were maintained in human erythrocytes (A^+/O^+) at 5% haematocrit in complete culture media RPMI 1640 [(Gibco) supplemented with 25 mM HEPES, 0.2% (w/v) D-glucose, 200 µM hypoxanthine, 0.2% (w/v) sodium bicarbonate, 24 µg/mL gentamicin and 0.5% (w/v) AlbuMAX II lipid-rich BSA)] under hypoxic conditions (90% N_2, 5% O_2 and 5% CO_2) at 37°C with agitation (Verlinden *et al.*, 2011). Asexual blood stage parasite stage-progression schizogony through ring, trophozoite and schizont development was monitored microscopically through Giemsa-stained thin smears (Fivelman *et al.*, 2007). Gametocytogenesis was induced (0.5% parasitaemia, 6% haematocrit) from the *Pf*NF54-PfS16-GFP-Luc line gifted by David Fidock, Columbia University, USA (Adjalley *et al.*, 2011) in glucose deprived complete RPMI media. Three days post induction, the haematocrit was decreased to 4%, with asexual parasite proliferation being inhibited through the addition of 50 mM N-acetylglucosamine (Sigma-Aldrich) in glucose supplemented complete RPMI media on days 5–10 post induction (Reader *et al.*, 2015). Progressive gametocyte development from early (stages I/II/III) to late (stages IV/V) stages were monitored microscopically using Giemsa-stained thin smears during daily media changes.

Asexual blood stage activity assays

Compounds were synthesised as previously reported (Cheuka *et al.*, 2018). Inhibitory activities of the compounds (10 mM stock solutions in 100% DMSO (w/v), Sigma-Aldrich) were determined against asexual *Pf*NF54 and *Pf*K1 parasites *in vitro* using SYBR Green I fluorescence as a antiproliferative assay (Smilkstein *et al.*, 2004, Verlinden *et al.*, 2011). An asexual ring-stage parasite population (1% parasitaemia and 1% haematocrit, > 95% D-sorbitol synchronised) were treated with a two-fold serial compound dilution in complete culture media (final DMSO concentration of < 0.1% (v/v)) in 96-well plates and incubated for 96 h at 37°C under 90% N_2, 5% CO_2, and 5% O_2 hypoxic conditions. Following incubation, parasite proliferation was determined by adding equal volumes of parasite suspension (100 µl) to SYBR Green I lysis buffer (0.2% of 10 000x SYBR Green I (Invitrogen), 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.008% saponin (w/v) and 0.08% Triton X-1000) and incubated in the dark for 1 h at room temperature. Fluorescence was quantified using the GloMaxR-Multi+ Detection System at 485/538 nm. The concentration of the compounds required to affect 50% parasite proliferation (IC_{50}) was determined using a non-linear curve fit (GraphPad Prism V6.0) normalised to maximum and minimum

inhibition. Data for each compound are from at least three independent biological replicates, each performed in technical triplicates. The resistance index (RI, IC_{50} ratio between drug resistant and sensitive strains) was calculated for the *Pf*K1 over *Pf*NF54 IC_{50} values.

Inhibition of gametocyte viability

Inhibitory activities of the compounds (10 mM stock solutions in 100% DMSO (w/v), Sigma-Aldrich) against early (50% stage II/50% stage III) and late-stage (40% stage IV/60% stage V) gametocytes at 2% gametocytaemia, 2% haematocrit were determined by treating gametocytes with 5 μM compound concentration in complete culture media (final DMSO concentration of < 0.1% (v/v)) for 48 h at 37°C under 90% N_2, 5% CO_2, and 5% O_2 hypoxic conditions using the *Pf*NF54-pfs16-GFP-Luc reporter line (Reader *et al.*, 2015, Coetzee *et al.*, 2020, van der Watt *et al.*, 2018). Dose–response curves against late-stage gametocytes (*Pf*NF54-pfs16-GFP-Luc reporter line) were determined using a two-fold serial compound dilution in complete culture media (final DMSO concentration of < 0.1% (v/v)) and incubated for 48 h at 37°C under 90% N_2, 5% CO_2, and 5% O_2 hypoxic conditions. The luciferase reporter assay was performed using equal volumes of parasite lysate (30 μl) and luciferin substrate (Promega luciferase assay system), with bioluminescence detected at a 10 s integration constant (GloMaxR-Multi+ Detection System) (Reader *et al.*, 2015). The IC_{50} was again determined using non-linear curve fit (GraphPad Prism V6.0) normalised to maximum and minimum inhibition and data are from at least three independent biological replicates, each performed in technical triplicates.

Mammalian cytotoxicity assays

Cytotoxity of compounds (10 mM stock solutions in 100% DMSO (w/v), Sigma-Aldrich) were determined against human caucasian hepatocellular carcinoma (HepG2) cells. The cells were maintained in DMEM media supplemented with 10% heat inactivated foetal bovine serum and 1% penicillin/streptomycin at 37°C under 5% CO_2 hypoxic conditions (Verlinden *et al.*, 2011). Cells were trypsinised once 80% confluency was reached using 0.25% Trypsin-EDTA and plated in 96-well plates (10 000 cells/well) and incubated for 24 h. Following incubation, cells were treated with 2 μM compound in complete media (final DMSO concentration of < 0.1% (v/v)) and incubated for 48 h at 37°C under 5% CO_2 hypoxic conditions. Following treatment cytotoxicity was determined using the lactate dehydrogenase (LDH) viability assay (Cytoselect Inc.) as per manufacturer's instructions by incubating 10 μl of the supernatant with 100 μl cytoselect reagent at 37°C for 30 min under 5% CO_2 hypoxic conditions. Cytotoxicity was quantified colorimetrically through absorbance at 450 nm using a SpectraMax Paradigm Multimode Detection Platform microplate reader (Molecular Devices) and normalised to maximum and minimum cytotoxicity. Assays were performed for a single biological repeat, in technical triplicates.

Structure function activity analysis

Structure–activity landscape index (SALI) was performed using Osiris Datawarrior V 5.2.1 software (www.openmolecules.org). The compound similarity/activity cliff analyses was performed with similarity based on the SMILES structure taking stereochemistry into account and separated based on the compound neighbour, with a Tanimoto similarity threshold at 86%.

RESULTS

3,6-diarylated imidazopyridazines inhibit asexual blood stage parasite proliferation

Initial *in vitro* asexual blood stage activities of 18 3,6-diarylated imidazopyridazines (Figure 1) was determined against *Pf*NF54 parasites using the SYBR Green I-based antiproliferative assay at 1 and 5 µM. All compounds, excluding **6**, inhibited parasite proliferation by more than 70% at 5 µM. However, only 9 compounds retained more than 50% inhibition at 1 µM (Figure 2a). These 9 compounds were selected for further dose–response evaluation (IC_{50}) against asexual blood stage parasites. All of these compounds displayed sub-micromolar activity against the drug sensitive *Pf*NF54 strain, with three compounds (**15**, **10** and **1**) being potent with IC_{50} values ≤ 100 nM (Figure 2b). These activities confirmed previously reported activities for 3,6-diaryalted imidazopyridazines as evaluated with a hypoxanthine incorporation assay (Cheuka *et al.*, 2018). Similarly, the majority of compounds did not show any cross-resistance against the multidrug resistant *Pf*K1 strain, as measured by the average resistance indices (RI, IC_{50} K1/NF54) for all compounds reported at < 3.9, compared to the RI of chloroquine as control at > 10 (Coertzen *et al.*, 2018). However, the most active compound, **15**, had a RI of > 72, indicating a potential cross-resistance of this compound with known antimalarials. Cytotoxicity screens against mammalian HepG2 cells indicated that the majority of the compounds did not negatively affect HepG2 cell viability at 2 µM except for **15** and **9**, both of which showed marginal cytotoxicity (> 84% viability remaining at 2 µM, Figure 2b).

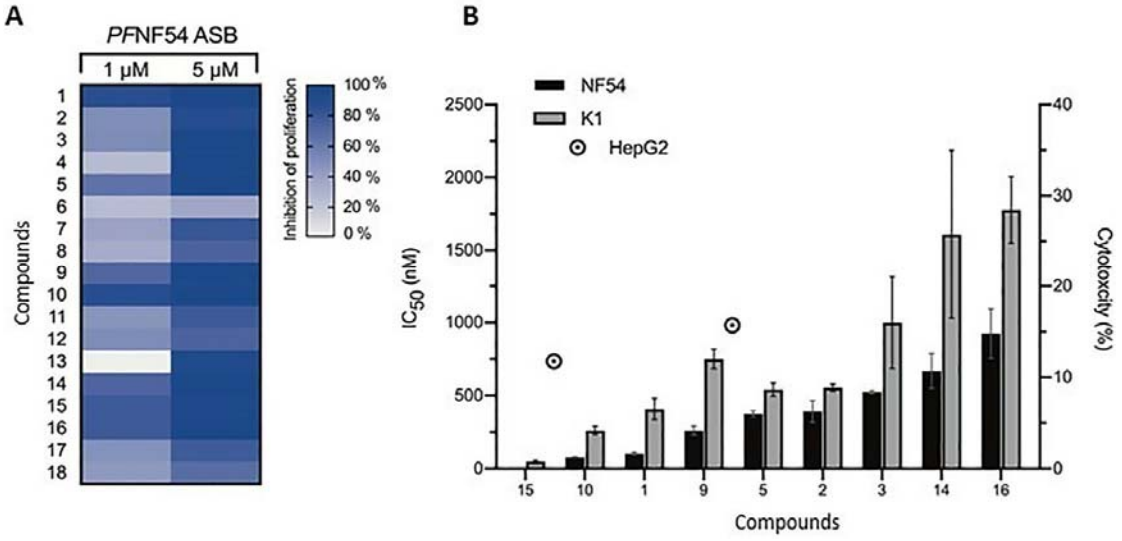


Figure 2. Activity of 3,6-diarylated imidazopyridazines against asexual parasites. (A) Inhibition (%) of asexual blood stage proliferation by 1 and 5 µM 3,6-diarylated imidazopyridazines after 96 h drug pressure, as measured with SYBR Green I fluorescence. Data are from a single biological repeat performed in technical triplicates. (B) Rank-ordered activities of imidazopyridazines against asexual parasites of *Pf*NF54 and *Pf*K1 determined with full dose-response analyses. Cytotoxicity (%) as determined with the LDH assay against HepG2 at 2 µM indicated on the secondary y-axis. Data are from a single biological repeat performed in technical triplicates. IC_{50} values are means from at least three independent biological repeats (n ≥ 3) performed in technical triplicates, ± S.E.

Frontrunner 3,6-diarylated imidazopyridazines are active against gametocytes

Initial activity screens against an early- (equal mix of stage II and III) and late-stage gametocyte (40% stage IV, 50% stage V) population were performed using the luciferase reporter viability assay at 5 μM. Although the majority of the compounds only had marginal activity against both early- and late-stage gametocytes, a linear relationship was present for the stage differentiated activities (Figure 3a), similar to previous reports for imidazopyridazines (van der Watt *et al.*, 2018). Only 5 compounds (**15, 5, 9, 10** and **3**) displayed > 50% inhibition, with only **15** and **10** showing > 70% inhibition at 1 μM. Compound **10** was the most potent with an IC_{50} of 1180.3 ± 117.8 nM, followed closely by **15** (IC_{50}: 1782.5 ± 699.3 nM) (Figure 3b). **9** was active at less than 5 μM (IC_{50}: 4231 ± 1647 nM) whereas **5** was inactive (IC_{50} > 5 μM). Taken together, the majority of the 3,6-diarylated imidazopyridazines are more selective towards the asexual blood stages but two compounds show promise as dual-active antiplasmodials.

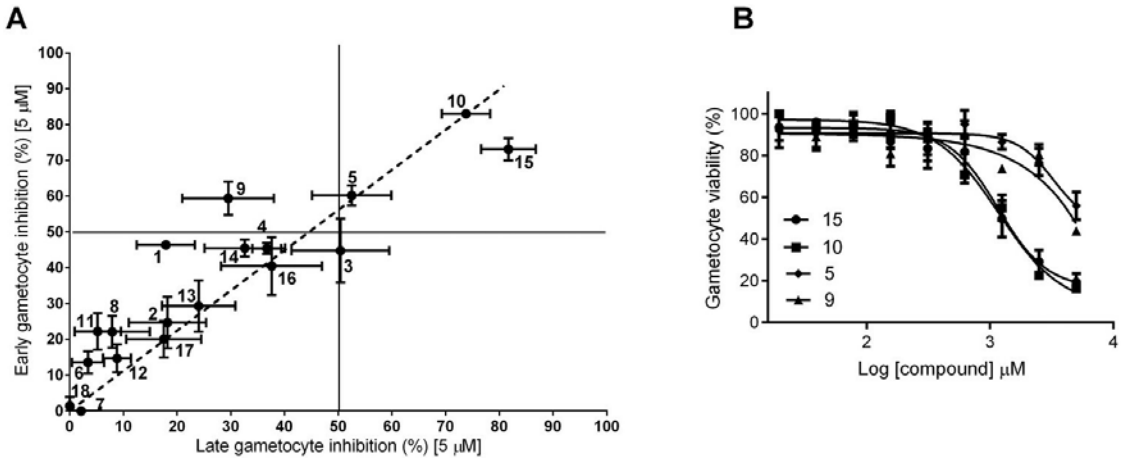


Figure 3. Activity determination of 3,6-diarylated imidazopyridazines against early- (II/III) and late-stage (IV/V) gametocytes and male gametes. (A) Inhibition (%) of early (EG, consisting of a 50% stage III, and 50% stage III population) and late stage gametocytes (LG, consisting of a 60% stage IV, and 40% stage V population) by 5 μM of 3,6-diarylated imidazopyridazines after 48 h drug pressure, as measured with the luciferase reporter assay. Data are from a single biological repeat performed in technical triplicates, ± S.D. (B) Dose-response curves of compounds showing > 50% inhibition at 5 μM against late stage gametocytes after 48 h drug pressure with the luciferase reporter assay. IC_{50} values are means from three independent biological repeats performed in technical triplicates, ± S.E.

Structure–activity landscape analysis of imidazopyridazines

To evaluate if the biological activity can be associated to the chemical features of the 3,6-diarylated imidazopyridazines, a structural-activity landscape was generated where the 3,6-diarylated imidazopyridazines was compared to precursor imidazopyridazines reported previously (van der Watt *et al.*, 2018) (Figure 4), with all the chemical substituents and activities within the different clusters being shown in Table 1. A highly-connected cluster contained near neighbours (**10** and **9**) of the lead compound, MMV652103, di-substituted with methylsulfonylphenyl in both positions and with IC_{50} values against asexual blood stages of 7 and 27 nM against gametocytes (Le Manach *et al.*, 2014a, van der Watt *et al.*, 2018) (Figure 4). **10** and **9** have similar solubility and biological profiles to MMV652103 with regard to asexual blood stage activities whilst also having appreciable gametocyte activities (Table 1). However, a change at the 3 position from a methylsulfonylphenyl (**10**) to

a methylsulfinylphenyl (**9**), detrimentally affected both asexual blood stage and gametocyte activity as seen in the ~4x fold drop in IC_{50}'s (Table 1). Interestingly, a change in the position of the methylsulfinyl group from the *meta* to the *para* position, as in **12**, on the phenyl ring at the 3 substitution, completely abolishes both asexual blood stage and gametocyte activity. Compounds **10** and **9** showed weak correlations to MMV669289 (IC_{50} against asexual blood stages of 95.9 and 1489 nM against gametocytes) (Le Manach *et al.*, 2014a, van der Watt *et al.*, 2018) also substituted with a methylsulfonylphenyl at the 3 position and MMV670654, bi-substituted with methylsulfinylphenyl (IC_{50} against asexual blood stages of 35 and 7993 nM against gametocytes) (Le Manach *et al.*, 2014a, van der Watt *et al.*, 2018). This indicates that the methylsulfonylphenyl group at the 3 position is generally responsible for good gametocytocidal activity (IC_{50} ~ 1000 nM).

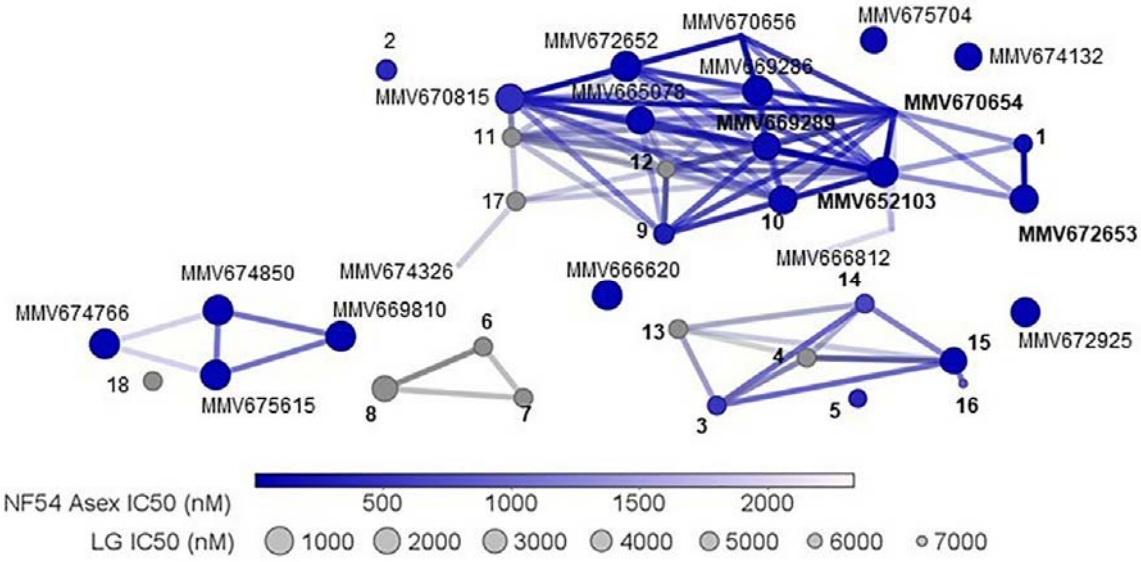


Figure 4. Inter-series SALI of precursor and 3,6-diarylated imidazopyridazines. Pairwise structural feature (SkelSphere) and late stage (LG, IV/V) analysis superimposed with *Pf*NF54 cliff analysis (Osiris DataWarrior) separately on each series at a similarity threshold of 86% in structural characteristics. Activity inclusions include IC_{50} values of asexual blood stage (ASB) and LG of the sub-micromolar to micromolar range. Activities and solubilities of compounds highlighted in bold are reflected in Table 1. Data are means from three independent biological repeats performed in technical triplicates. ASB and LG data for precursor imidazopyridazines from (van der Watt *et al.*, 2018).

The 6 position methylsulfonylphenyl substituted near neighbour of MMV652103, MMV672653 (IC_{50}s of 35.8 and 907 nM against asexual blood stage and late-stage gametocytes, respectively (van der Watt *et al.*, 2018)), is closely related to **1** that contains a benzoic acid group at position 3 (Table 1). This change essentially shifts the activity profile of the compound fully into having only asexual blood stage activity (IC_{50} = 104 nM) with complete loss of gametocyte activity (IC_{50} > 5 μM).

The remainder of the 3,6-diarylated imidazopyridazines formed individual clusters of either a non-active cluster of substituted methylsulfonylanilines/methylsulfinylanilines (**8**, **6** and **7**, Table 1), where improved solubility (≥ 60 μM) was to the cost of activity, or an asexual blood stage active cluster of substituted methylsulfinylphenyl and diethyl[(3-fluorophenyl)methyl]amines (**3, 13, 15, 14, 4** and **16**, Table 1). Changes in the sulfone position on the benzene in the 6 position, together with introduction of an additional amine on the core imidazopyridazine is not tolerated at all (compounds **6, 7** and **8)**. Within the asexual

blood stage active cluster, **15** and **16** contain the same 3 group substituents. However, they differ at the 6 group with a biaryl group for **15** and an amine linker to the pyridazine for **16**, implying that the biaryl group for **15** is essential for activity. Despite compounds **15** and **16** showing significantly improved solubility (200 μM at pH 6.5, Table 1) the biaryl group on compound **15** could be indicative of human toxicity as evidenced by decreased hERG activity (0.59 ± 0.02 μM) compared to the lead imidazopyridazine precursor, MMV652103 (3.61 ± 0.62 μM) (Cheuka *et al.*, 2018, Le Manach *et al.*, 2014b) (Table 1) and marginal HepG2 cytotoxicity (11.8% at 2 μM). Whereas, despite the significant loss in activity, the pyridazine amine linker of compound **16** is more ideal, with improved hERG IC_{50} (7.83 ± 1.21 μM, Table 1) activity and no HepG2 cytotoxicity (0% at 2 μM).

DISCUSSION

Current antimalarials provide chemotherapeutic protection by effectively clearing pathogenic asexual blood stage parasites, classifying them as target candidate profile 1 (TCP1) compounds. Although clearance of asexual blood stages is curative of the disease, the remaining transmissible gametocyte reservoirs are responsible for the continuous spread of the disease. Therefore, malaria elimination strategies have prioritised the development of compounds that target the sexually differentiated gametocyte stages as a means to block disease transmission (TCP5) (Burrows *et al.*, 2017). Combining compounds with either specific profiles associated with TCP1 or TCP5 can provide an all-encompassing solution to effectively eliminate malaria, thereby not only being curative of the pathogenic stages and blocking transmission, but also slowing down the rate of emergence of drug resistance. However, the development of TCP5-selective compounds has raised a unique set of pharmacokinetic (PK) challenges as compared to the development of TCP1 compounds only (Birkholtz *et al.*, 2016). Development of the *Pf*PI4 K inhibitors, pre-clinical candidate aminopyrazine (UCT943) (Brunschwig *et al.*, 2018) and the clinical candidate aminopyridine (MMV390048) (Paquet *et al.*, 2017), which have almost equipotent TCP1 and TCP5 activity, validated kinase inhibitors as a novel class of potent dual-active compounds.

Of the 18 3,6-diarylated imidazopyridazines tested here, all the compounds showed sub- to micromolar activities against asexual blood stages, similar to those previously reported (Cheuka *et al.*, 2018), and highlighting the potential of the series as TCP1 actives. Imidazopyridazines have several favourable characteristics in relation to other kinase inhibitors such as 2-aminopyridines including a more rapid speed of action (within 48 h) against asexual blood stages, with some compounds even targeting early-stage gametocytes within 24 h (van der Watt *et al.*, 2018), compared to 2-aminopyridines with a slower speed of action (González Cabrera *et al.*, 2018). Therefore, fast-acting imidazopyridazines at an effective dose can ensure clearance of asexual blood stages prior to gametocytogenesis and in this way still be effective without directly targeting gametocytes.

The majority of the compounds on average had more than 10x decrease in activity against late-stage gametocytes. This is a profile that has been seen for several classes of compounds (Plouffe *et al.*, 2016, Coertzen *et al.*, 2018), but is unlike that observed for the precursor imidazopyridazine scaffolds (van der Watt *et al.*, 2018) where several compounds retained activity against late-stage gametocytes. Modifications made to solve solubility and cardiotoxicity risks therefore were to the detriment of gametocytocidal activity, with only 2 compounds showing promise as dual-active candidates. Interestingly though, the 3,6-diarylated imidazopyridazines showed a linear correlation in their activities across early- and late-stage gametocytes, similar to precursor imidazopyradazines. This is in contrast to

compounds from the 2-aminopyridine scaffold, which showed selectivity towards late-stage gametocytes (van der Watt *et al.*, 2018). This could point to differences in mode of action in these series against gametocytes. Indeed, imidazopyridazines do target *Pf*PI4 K (González Cabrera *et al.*, 2018), similarly to the 2-aminopyridines (Paquet *et al.*, 2017). However, imidazopyridazines also target additional kinases, such as *P. falciparum* calcium-dependent protein kinase 1 (*Pf*CDPK1) (Ansell *et al.*, 2014) and *P. falciparum* guanosine monophosphate (cGMP)-dependent protein kinase G (*Pf*PKG) (Alam *et al.*, 2015, Green *et al.*, 2016). Both of these kinases are expressed (van Biljon *et al.*, 2019) and are functionally important in gametocytes and other sexual stages (e.g. ookinetes) (Brochet *et al.*, 2014), which could explain the differentiation of this series and activity against both early- and late-stage gametocytes. Interestingly, a recent study by Cheuka *et al.* (accepted to ACS Infectious disease, manuscript ID: id-2020-004818.R2) showed that similar analogues to the 3,6-diarylated compounds evaluated here, 3,6-diphenylated imidazopyridazine analogues, target both *Pf*PI4 K and *Pf*PKG. It would therefore be interesting to validate the targets for the 3,6-diarylated imidazopyridazines and the importance thereof for multiple life cycle stages in *P. falciparum*.

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REFERENCES

Adjalley, S.H., Johnston, G.L., Li, T., Eastman, R.T., Ekland, E.H., Eappen, A.G., Richman, A., Sim, B.K., Lee, M.C., Hoffman, S.L. & Fidock, D.A. 2011. Quantitative assessment of Plasmodium falciparum sexual development reveals potent transmission-blocking activity by methylene blue. *Proceedings of the National Academy of Sciences of the United States of America* 108: 1214–1223.

Alam, M.M., Solyakov, L., Bottrill, A.R., Flueck, C., Siddiqui, F.A., Singh, S., Mistry, S., Viskaduraki, M., Lee, K., Hopp, C.S., Chitnis, C.E., Doerig, C., Moon, R.W., Green, J.L., Holder, A.A., Baker, D.A. & Tobin, A.B. 2015. Phosphoproteomics reveals malaria parasite Protein Kinase G as a signalling hub regulating egress and invasion. *Nature Communications* 6: 7285.

Anamika, K., Srinivasan, N. & Krupa, A. 2005. A genomic perspective of protein kinases in *Plasmodium falciparum*. *Proteins: Structure, Function, and Bioinformatics* 58: 180–189.

Ansell, K.H., Jones, H.M., Whalley, D., Hearn, A., Taylor, D.L., Patin, E.C., Chapman, T.M., Osborne, S.A., Wallace, C., Birchall, K., Large, J., Bouloc, N., Smiljanic-Hurley, E., Clough, B., Moon, R.W., Green, J.L. & Holder, A.A. 2014. Biochemical and Antiparasitic Properties of Inhibitors of the Calcium-Dependent Protein Kinase PfCDPK1. *Antimicrobial Agents and Chemotherapy* 58: 6032–6043.

Birkholtz, L.-M., Coetzer, T.L., Mancama, D., Leroy, D. & Alano, P. 2016. Discovering New Transmission-Blocking Antimalarial Compounds: Challenges and Opportunities. *Trends in Parasitology* 32: 669–681.

Brochet, M., Collins, M.O., Smith, T.K., Thompson, E., Sebastian, S., Volkmann, K., Schwach, F., Chappell, L., Gomes, A.R., Berriman, M., Rayner, J.C., Baker, D.A., Choudhary, J. & Billker, O. 2014. Phosphoinositide metabolism links cGMP-dependent protein kinase G to essential Ca^{2+} signals at key decision points in the life cycle of malaria parasites. *PLoS Biol* 12: e1001806.

Brunschwig, C., Lawrence, N., Taylor, D., Abay, E., Njoroge, M., Basarab, G.S., Le Manach, C., Paquet, T., Cabrera, D.G., Nchinda, A.T., De Kock, C., Wiesner, L., Denti, P., Waterson, D., Blasco, B., Leroy, D., Witty, M.J., Donini, C., Duffy, J., Wittlin, S., White, K.L., Charman, S.A., Jiménez-Díaz, M.B., Angulo-Barturen, I., Herreros, E., Gamo, F.J., Rochford, R., Mancama, D., Coetzer, T.L., Van Der Watt, M.E., Reader, J., Birkholtz, L.M., Marsh, K.C., Solapure, S.M., Burke, J.E., Mcphail, J.A., Vanaerschot, M., Fidock, D.A., Fish, P.V., Siegl, P., Smith, D.A., Wirjanata, G., Noviyanti, R., Price, R.N., Marfurt, J., Silue, K.D., Street, L.J. & Chibale, K. 2018. UCT943, a Next-Generation Plasmodium falciparum PI4 K Inhibitor Preclinical Candidate for the Treatment of Malaria. *Antimicrob Agents Chemother* 62 (9): e00012–18.

Burrows, J.N., Duparc, S., Gutteridge, W.E., Hooft Van Huijsduijnen, R., Kaszubska, W., Macintyre, F., Mazzuri, S., Möhrle, J.J. & Wells, T.N.C. 2017. New developments in anti-malarial target candidate and product profiles. *Malaria Journal* 16: 26.

Cheuka, P.M., Lawrence, N., Taylor, D., Wittlin, S. & Chibale, K. 2018. Antiplasmodial imidazopyridazines: structure-activity relationship studies lead to the identification of analogues with improved solubility and hERG profiles. *RSC Medicinal Chemistry - Royal Society of Chemistry* 9: 1733–1745.

Coertzen, D., Reader, J., Van Der Watt, M., Nondaba, S.H., Gibhard, L., Wiesner, L., Smith, P., D'alessandro, S., Taramelli, D., Wong, H.N., Du Preez, J.L., Wu, R.W.K., Birkholtz, L.-M. & Haynes, R.K. 2018. Artemisone and artemiside - potent pan-reactive antimalarial agents that also synergize redox imbalance in P. falciparum transmissible gametocyte stages. *Antimicrob. Agents Chemother* 62: e02214–17.

Coetzee, N., Von Grüning, H., Opperman, D., Van Der Watt, M., Reader, J. & Birkholtz, L.-M. 2020. Epigenetic inhibitors target multiple stages of Plasmodium falciparum parasites. *Scientific Reports* 10: 2355.

Duffy, S. & Avery, V.M. 2012. Development and optimization of a Novel 384-well anti-malarial imaging assay validated for high-throughput screening. *The American Journal of Tropical Medicine and Hygiene* 86: 84–92.

Fivelman, Q.L., Mcrobert, L., Sharp, S., Taylor, C.J., Saeed, M., Swales, C.A., Sutherland, C.J. & Baker, D.A. 2007. Improved synchronous production of Plasmodium falciparum gametocytes in vitro. *Molecular and Biochemical Parasitology* 154: 119–123.

González Cabrera, D., Horatscheck, A., Wilson, C.R., Basarab, G., Eyermann, C.J. & Chibale, K. 2018. Plasmodial Kinase inhibitors: License to cure? *Journal of Medicinal Chemistry* 61: 8061–8077.

Green, J.L., Moon, R.W., Whalley, D., Bowyer, P.W., Wallace, C., Rochani, A., Nageshan, R.K., Howell, S.A., Grainger, M., Jones, H.M., Ansell, K.H., Chapman, T.M., Taylor, D.L., Osborne, S.A., Baker, D.A., Tatu, U. & Holder, A.A. 2016. Imidazopyridazine inhibitors of *Plasmodium falciparum* calcium-dependent protein Kinase 1 also target cyclic GMP-dependent protein kinase and heat shock Protein 90 to kill the parasite at different stages of intracellular development. *Antimicrobial Agents and Chemotherapy* 60: 1464–1475.

Kini, S.G., Garg, V., Prasanna, S., Rajappan, R. & Mubeen, M. 2017. Protein kinases as drug targets in human and animal diseases. *Current Enzyme Inhibition* 13: 99–108.

Le Manach, C., Gonzàlez Cabrera, D., Douelle, F., Nchinda, A.T., Younis, Y., Taylor, D., Wiesner, L., White, K.L., Ryan, E., March, C., Duffy, S., Avery, V.M., Waterson, D., Witty, M.J., Wittlin, S., Charman, S.A., Street, L.J. & Chibale, K. 2014a. medicinal chemistry optimization of antiplasmodial imidazopyridazine hits from high throughput screening of a softfocus kinase library: part 1. *Journal of Medicinal Chemistry* 57: 2789–2798.

Le Manach, C., Paquet, T., Brunschwig, C., Njoroge, M., Han, Z., Gonzàlez Cabrera, D., Bashyam, S., Dhinakaran, R., Taylor, D., Reader, J., Botha, M., Churchyard, A., Lauterbach, S., Coetzer, T.L., Birkholtz, L.-M., Meister, S., Winzeler, E.A., Waterson, D., Witty, M.J., Wittlin, S., Jiménez-Díaz, M.-B., Santos Martínez, M., Ferrer, S., Angulo-Barturen, I., Street, L.J. & Chibale, K. 2015a. A Novel Pyrazolopyridine with in vivo activity in Plasmodium berghei- and Plasmodium falciparum-infected mouse models from structure–activity relationship studies around the core of recently identified antimalarial imidazopyridazines. *Journal of Medicinal Chemistry* 58: 8713–8722.

Le Manach, C., Paquet, T., Brunschwig, C., Njoroge, M., Han, Z., Gonzàlez Cabrera, D., Bashyam, S., Dhinakaran, R., Taylor, D., Reader, J., Botha, M., Churchyard, A., Lauterbach, S., Coetzer, T.L., Birkholtz, L.M., Meister, S., Winzeler, E.A., Waterson, D., Witty, M.J., Wittlin, S., Jiménez-Díaz, M.B., Santos Martínez, M., Ferrer, S., Angulo-Barturen, I., Street, L.J. & Chibale, K. 2015b. A novel pyrazolopyridine with in vivo activity in plasmodium berghei- and plasmodium falciparum-infected mouse models from structure-activity relationship studies around the core of recently identified antimalarial imidazopyridazines. *J Med Chem* 58: 8713–8722.

Le Manach, C., Paquet, T., Gonzàlez Cabrera, D., Younis, Y., Taylor, D., Wiesner, L., Lawrence, N., Schwager, S., Waterson, D., Witty, M.J., Wittlin, S., Street, L.J. & Chibale, K. 2014b. Medicinal chemistry optimization of antiplasmodial imidazopyridazine hits from high throughput screening of a softfocus kinase library: part 2. *Journal of Medicinal Chemistry* 57: 8839–8848.

Lemercier, G., Fernandez-Montalvan, A., Shaw, J.P., Kugelstadt, D., Bomke, J., Domostoj, M., Schwarz, M.K., Scheer, A., Kappes, B. & Leroy, D. 2009. Identification and characterization of novel small molecules as potent inhibitors of the plasmodial calcium-dependent protein kinase 1. *Biochemistry* 48: 6379–6389.

Matsumoto, S., Miyamoto, N., Hirayama, T., Oki, H., Okada, K., Tawada, M., Iwata, H., Nakamura, K., Yamasaki, S., Miki, H., Hori, A. & Imamura, S. 2013. Structure-based design, synthesis, and evaluation of imidazo[1,2-b]pyridazine and imidazo[1,2-a]pyridine derivatives as novel dual c-Met and VEGFR2 kinase inhibitors. *Bioorganic & Medicinal Chemistry* 21: 7686–7698.

Mcnamara, C. W., Lee, M. C. S., Lim, C. S., Lim, S. H., Roland, J., Nagle, A., Simon, O., Yeung, B. K. S., Chatterjee, A. K., Mccormack, S. L., Manary, M. J., Zeeman, A.-M., Dechering, K. J., Kumar, T. R. S., Henrich, P. P., Gagaring, K., Ibanez, M., Kato, N., Kuhen, K. L., Fischli, C., Rottmann, M., Plouffe, D. M., Bursulaya, B., Meister, S., Rameh, L., Trappe, J., Haasen, D., Timmerman, M., Sauerwein, R. W., Suwanarusk, R., Russell, B., Renia, L., Nosten, F., Tully, D. C., Kocken, C. H. M., Glynne, R. J., Bodenreider, C., Fidock, D. A., Diagana, T. T. & Winzeler, E. A. 2013. Targeting Plasmodium PI(4)K to eliminate malaria. *Nature* 504: 248-253.

Moran, D.B., Powell, D.W. & Albrigh, J.D. 1986. Imidazo[1,2-b] Pyridazine.

Osborne, S., Chapman, T., Large, J., Bouloc, N. & Wallace, C. 2011. Fused Heterocyclic Compounds for Use in the Treatment of Malaria.

Paquet, T., Le Manach, C., Cabrera, D.G., Younis, Y., Henrich, P.P., Abraham, T.S., Lee, M.C.S., Basak, R., Ghidelli-Disse, S., Lafuente-Monasterio, M.J., Bantscheff, M., Ruecker, A., Blagborough, A.M., Zakutansky, S.E., Zeeman, A.M., White, K.L., Shackleford, D.M., Mannila, J., Morizzi, J., Scheurer, C., Angulo-Barturen, I., Martínez, M.S., Ferrer, S., Sanz, L.M., Gamo, F.J., Reader, J., Botha, M., Dechering, K.J., Sauerwein, R.W., Tungtaeng, A., Vanachayangkul, P., Lim, C.S., Burrows, J., Witty, M.J., Marsh, K.C., Bodenreider, C., Rochford, R., Solapure, S.M., Jiménez-Díaz, M.B., Wittlin, S., Charman, S.A., Donini, C., Campo, B., Birkholtz, L.M., Hanson, K.K., Drewes, G., Kocken, C.H.M., Delves, M.J., Leroy, D., Fidock, D.A., Waterson, D., Street, L.J. & Chibale, K. 2017. Antimalarial efficacy of MMV390048, an inhibitor of Plasmodium phosphatidylinositol 4-kinase. *Sci Transl Med* 9 (387): eaad9735.

Plouffe, D.M., Wree, M., Du, A.Y., Meister, S., Li, F., Patra, K., Lubar, A., Okitsu, S.L., Flannery, E.L., Kato, N., Tanaseichuk, O., Comer, E., Zhou, B., Kuhen, K., Zhou, Y., Leroy, D., Schreiber, S.L., Scherer, C.A., Vinetz, J. & Winzeler, E.A. 2016. High-Throughput Assay and Discovery of Small Molecules that Interrupt Malaria Transmission. *Cell Host & Microbe* 19: 114–126.

Reader, J., Botha, M., Theron, A., Lauterbach, S.B., Rossouw, C., Engelbrecht, D., Wepener, M., Smit, A., Leroy, D., Mancama, D., Coetzer, L.C. & Birkholtz, L. 2015. Nowhere to hide: interrogating different metabolic parameters of Plasmodium falciparum gametocytes in a transmission blocking drug discovery pipeline towards malaria elimination. *Malaria Journal* 14: 213.

Sahu, N.K., Shahi, S., Sharma, M.C. & Kohli, D.V. 2011. QSAR studies on imidazopyridazine derivatives as PfPK7 inhibitors. *Molecular Simulation* 37: 752–765.

Singh, K., Okombo, J., Brunschwig, C., Ndubi, F., Barnard, L., Wilkinson, C., Njogu, P.M., Njoroge, M., Laing, L., Machado, M., Prudêncio, M., Reader, J., Botha, M., Nondaba, S., Birkholtz, L.M., Lauterbach, S., Churchyard, A., Coetzer, T.L., Burrows, J.N., Yeates, C.,

Denti, P., Wiesner, L., Egan, T.J., Wittlin, S. & Chibale, K. 2017. Antimalarial Pyrido[1,2-a]benzimidazoles: lead optimization, parasite life cycle stage profile, mechanistic evaluation, killing kinetics, and in vivo oral efficacy in a mouse model. *J Med Chem* 60: 1432–1448.

Smilkstein, M., Sriwilaijaroen, N., Kelly, J.X., Wilairat, P. & Riscoe, M. 2004. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. *Antimicrobial Agents and Chemotherapy* 48: 1803–1806.

Van Biljon, R., Van Wyk, R., Painter, H.J., Orchard, L., Reader, J., Niemand, J., LlináS, M. & Birkholtz, L.M. 2019. Hierarchical transcriptional control regulates Plasmodium falciparum sexual differentiation. *BMC Genomics* 20: 920.

Van Der Watt, M.E., Reader, J., Churchyard, A., Nondaba, S.H., Lauterbach, S.B., Niemand, J., Abayomi, S., Van Biljon, R.A., Connacher, J.I., Van Wyk, R.D.J., Le Manach, C., Paquet, T., González Cabrera, D., Brunschwig, C., Theron, A., Lozano-Arias, S., Rodrigues, J.F.I., Herreros, E., Leroy, D., Duffy, J., Street, L.J., Chibale, K., Mancama, D., Coetzer, T.L. & Birkholtz, L.-M. 2018. Potent Plasmodium falciparum gametocytocidal compounds identified by exploring the kinase inhibitor chemical space for dual active antimalarials. *Journal of Antimicrobial Chemotherapy* 73: 1279–1290.

Verlinden, B.K., Niemand, J., Snyman, J., Sharma, S.K., Beattie, R.J., Woster, P.M. & Birkholtz, L. 2011. Discovery of novel alkylated (bis)urea and (bis)thiourea polyamine analogues with potent antimalarial activities. *Journal of Medicinal Chemistry* 54: 6624–6633.

Ward, P., Equinet, L., Packer, J. & Doerig, C. 2004. Protein kinases of the human malaria parasite *Plasmodium falciparum*: the kinome of a divergent eukaryote. *BMC Genomics* 5: 79–79.

WHO. 2019. World Malaria Report 2019.

Zhang, J., Yang, P.L. & Gray, N.S. 2009. Targeting cancer with small molecule kinase inhibitors. *Nature Reviews Cancer* 9: 28.