The Human Chk1 Inhibitor CHIR-124 Shows Multistage Activity Against *Plasmodium falciparum* via Dual Inhibition of *Pf*Ark1 and Hemozoin Formation

Kathryn J. Wicht,[1][2][3] John G. Woodland,[1][2][3] Larnelle F. Garnie,[3] Henrico Langeveld,[4][5], Dale Taylor,[1] Luiz C. Godoy,[6] Charisse Flerida A. Pasaje,[6] Mariana Laureano de Souza,[7] Jair L. Siqueira-Neto,[7] Sonja Ghidelli-Disse,[8] Maria Jose Lafuente-Monasterio,[9] Francisco-Javier Gamo,[9] Dina Coertzen,[4] Janette Reader,[4] Mariëtte van der Watt,[4] Jessica L. Bridgford,[10] Gareth Girling,[11] Rachael Coyle,[11][12] Christian Scheurer,[13][14] Sergio Wittlin,[13][14] Marcus C.S. Lee,[11][12] Till S. Voss,[13][14] Elizabeth A. Winzeler,[7] David A. Fidock,[10] Jacquin C. Niles,[6] Lyn-Marié Birkholtz,[4][5] Lauren B. Coulson,[1] and Kelly Chibale*[1][2][3]

[1] Holistic Drug Discovery and Development (H3D) Centre, University of Cape Town, Rondebosch 7701, South Africa.

[2] South African Medical Research Council Drug Discovery and Development Research Unit, Department of Chemistry and Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Observatory 7925, South Africa.

[3] Department of Chemistry, University of Cape Town, Rondebosch, 7701, South Africa.

[4] Department of Biochemistry, Genetics and Microbiology, Institute for Sustainable Malaria Control, University of Pretoria, Hatfield 0028, South Africa.

[5] Department of Biochemistry, Stellenbosch University, Stellenbosch, Matieland 7602, South Africa.

[6] Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.

[7] Department of Pediatrics and Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, La Jolla, CA, USA.

[8] Cellzome GmbH, a GSK Company, Heidelberg, Germany.

[9] GlaxoSmithKline, Tres Cantos Medicines Development Campus, Madrid, Spain.

[10] Department of Microbiology and Immunology, and Center for Malaria Therapeutics and Antimicrobial Resistance, Division of Infectious Diseases, Department of Medicine, Columbia University Irving Medical Center, New York, NY, USA.

[11] Wellcome Sanger Institute, Wellcome Genome Campus, Hinxton, UK.

[12] Division of Biological Chemistry and Drug Discovery, Wellcome Centre for Anti-Infectives Research, University of Dundee, Dundee, UK.

[13] Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, 4123 Allschwil, Switzerland

[14] University of Basel, 4001 Basel, Switzerland

*** Corresponding author:** Kelly Chibale; Orcid.org/0000-0002-1327-4727; kelly.chibale@uct.ac.za

Keywords: Antiprotozoal agents • biological activity • hemozoin • kinase • *Plasmodium*

Additional supporting information can be found online in the Supporting Information section

Abstract: The high burden of malaria and growing resistance to frontline antimalarials demand new drugs with reduced propensities for generating resistance. An attractive approach to identifying chemical hits as starting points for antimalarial drug discovery involves the repositioning and chemical optimization of compounds used in other disease areas that are active against the human malaria parasite *Plasmodium falciparum*. Here we show that the human checkpoint kinase 1 (Chk1) inhibitor CHIR-124 is active *in vitro* against both drug-sensitive and drug-resistant asexual blood stage parasites and competitively binds to several *Plasmodium* kinases, including *P. falciparum* aurora-related kinase-1 (*Pf*Ark1). The compound also shows moderate activity against both the liver and gametocyte forms of the parasite. Further target investigation for CHIR-124 via conditional knockdown experiments confirmed that *Pf*Ark1 is implicated in its parasiticidal activity. Notably, CHIR-124 also inhibits β-hematin (synthetic hemozoin) formation and causes a dose-dependent increase in free heme that correlates with inhibition of parasite growth. These findings suggest that polypharmacology is involved in the activity of CHIR-124 against *P. falciparum* via the dual inhibition of *Plasmodium Pf*Ark1 and hemozoin formation, both essential for parasite proliferation. This is further supported by *in vitro* drug combination experiments, morphological studies and resistance generation attempts. This study demonstrates the feasibility of designing dual *Plasmodium* kinase/hemozoin formation inhibitors active against resistant strains with decreased resistance risks in the fight against malaria.

Introduction

The mosquito-borne disease of malaria is caused by protozoan parasites of the *Plasmodium* genus, of which *P. falciparum* is the most pervasive and deadly to humans.[1] *P. falciparum* parasites have a complex life cycle involving over ten different morphological forms, with the asexual blood stage (ABS) in the human host giving rise to clinical pathology in malaria patients.[2,3] Hypnozoites (liver stage) and gametocytes (transmission stage) are also biologically relevant life cycle stages within the human host. However, rapidly reducing the parasite burden at the ABS stage is the primary objective of most therapeutic antimalarials.[4] Drugs active against the ABS are categorized by the Medicines for Malaria Venture (MMV) Target Candidate Profiles (TCP) as TCP1.[5,6] Nevertheless, for malaria eradication, strategies that break the cycle of disease transmission need to be considered.[7] TCP1 molecules should ideally be paired with a partner drug also targeting the liver-stage hypnozoites for prophylactic treatment (TCP4) or the transmission-blocking stage gametocytes (TCP5) in addition to the ABS.[7,8]

Unfortunately, malaria mortality and incidence rates have increased since 2015, largely due to the slowed progress of the World Health Organization (WHO) Global Malaria Programme and further setbacks from the Covid-19 pandemic.[9] The WHO reported an estimated 263 million malaria cases and 597,000 malaria deaths worldwide in 2023, and highlighted that 94% of the disease burden is in sub-Saharan Africa.[10] With partial drug resistance to essential frontline regimens such as the artemisinin (ART) derivatives and their partner drugs on the rise, this is a critical time for discovering new antimalarials.[11,12] Historically, developing such therapies has been an immensely time-consuming and expensive task, with the discovery phase requiring that drug candidates meet strict criteria to progress to clinical development.[13] This has led to high attrition rates and prompted the desire for more efficient strategies for identifying new drug targets and drug molecules, particularly those with a low risk for generating resistance in parasite populations.[14]

One approach offering potential advantages compared with traditional antimalarial drug development is drug repurposing or repositioning, whereby previously approved compounds that have already been through clinical trials and safety studies are evaluated for their efficacy in new indications.[15,16] Approximately one-third of medicine approvals in recent years are owed to drug repurposing programs[17] and several groups have reported on this approach in the context of malaria.[18-21] While the use of de-risked compounds might suggest shorter development timelines, additional important aspects remain, such as regulatory challenges, as well as the need to understand disease-relevant dosing, safety and drug targets.[15]

Identifying potential target-focused molecules for repositioning is facilitated in cases where there is an ortholog target protein family in *Plasmodium*. To this end, repositioning human kinase inhibitors for malaria provides a promising avenue for drug development. Several *Plasmodium* kinases have been identified as favorable antimalarial targets, given their druggability and essentiality during more than one stage of the parasite life cycle.[22] Kinases are central to signal transduction pathways and play critical physiological roles in cell growth and proliferation.[23] Given the pivotal role of kinase inhibitors in non-communicable diseases, a wealth of structural data supports the design and optimization of kinase inhibitors.[24]

Only one *Plasmodium* kinase inhibitor, MMV390048, which targets phosphatidylinositol 4-kinase type III beta (*Pf*PI4KIIIβ), has reached the clinical stage of development for malaria so far.[25] Nevertheless, multiple opportunities exist for exploitation of this class of drug targets. Given the conserved nature of the ATP-binding site across the kinase superfamily, kinase inhibitors have the potential to display polypharmacology, which is advantageous for minimizing the generation of resistance.[22,26] Although selectivity for *Plasmodium* relative to human kinases is a key challenge for kinase-focused malaria drug discovery, identifying compounds that bind to *Plasmodium* kinases is an appealing approach, not only for finding new scaffolds with antimalarial activity but also for discovering kinase-inhibiting tool compounds.

Here we disclose the identification of the human Chk1 inhibitor CHIR-124 from a phenotypic ABS screen of clinical or pre-clinical anticancer human kinase inhibitors. We show that CHIR-124 possesses multistage activity against *P. falciparum* as well as retained potency against drug-resistant strains. Target identification via *Plasmodium* kinases captured on Kinobeads as well as biochemical, conditional knockdown, intracellular heme fractionation, morphological and drug combination studies reveal polypharmacology involved in the mode of action of CHIR-124.

Results and Discussion

CHIR-124 Identified via Kinobead Assays with Multistage Antiplasmodial Activity

A set of clinical human kinase inhibitors in clinical or pre-clinical development for the treatment of cancer was screened against *P. falciparum*. The human checkpoint 1 (Chk1) inhibitor, CHIR-124 (GSK3210608, **Figure 1A**), was identified with activity <1 μM against *Pf*3D7 (wild-type, drug sensitive) parasites. In humans, Chk1 is a serine/threonine kinase that regulates the G2/M cell cycle checkpoints and delays cell cycle progression in response to DNA damage.[27] Disrupting Chk1-mediated checkpoints renders tumor cells more susceptible to DNA damage and is, therefore, a convenient target of chemotherapy for cancers.[28] CHIR-124 has antitumor properties when used in combination with topoisomerase I poisons.[29]

To identify potential kinase targets in *Plasmodium*, CHIR-124 was screened against 75 *Plasmodium* kinases captured onto Kinobeads from ABS parasite lysates using a competitive pulldown assay (**Figure 1A**).[30,31] A total of eight *Plasmodium* kinases were competed off the Kinobeads by CHIR-124 at 10 μM with a $\log_2$ fold-change below the cutoff of -1 relative to the vehicle control (**Supplementary Figure S1**). Of these, five showed half-maximal inhibitory concentration (IC_{50}) values below 1 μM with apparent dissociation constants K_d^{app} below 0.7 μM. They included the calcium-dependent protein kinase 1 (*Pf*CDPK1, PF3D7_0217500, K_d^{app} 0.04 μM), 3-phosphoinositide-dependent protein kinase 1 (*Pf*PDK1, PF3D7_1121900, K_d^{app} 0.1 μM), aurora-related kinase 1 (*Pf*Ark1, PF3D7_0605300, K_d^{app} 0.17 μM) and the cAMP-dependent protein kinase regulatory/catalytic subunits (*Pf*PKAr/c, PF3D7_1223100/PF3D7_0934800, K_d^{app} 0.61 μM) (**Figure 1B, Supplementary Table S1**). Given that *Plasmodium* kinases are generally expressed across multiple stages of the parasite life cycle, albeit to differing degrees,[32] the potential of CHIR-124 to inhibit several *Plasmodium* kinases prompted us to evaluate its activity across the three different host life cycle stages.

For the dose-dependent growth inhibition of *P. falciparum* by CHIR-124 across the life cycle stages, we profiled CHIR-124 against *in vitro* cultures of the ABS, both immature and late-stage gametocytes, and the liver stage. These assays confirmed the sub-micromolar activity of CHIR-124 against ABS parasites in the drug-sensitive strain (**Figure 1C, Table 1** and **Supplementary Table S2**). Moderate activity was also observed against immature and late-stage gametocytes, in the low micromolar range, as well as against liver stages, in the sub-micromolar range (**Table 1** and **Supplementary Table S3**). Furthermore, CHIR-124 displayed roughly 14-fold selectivity for liver stage parasites relative to the HepG2 mammalian liver cell line (**Table 1** and **Supplementary Table S3**). The multistage activity of CHIR-124 suggests that at least one of its intracellular targets is biologically crucial at each life cycle stage tested.

We also profiled CHIR-124 against ABS drug-resistant strains, including the Southeast Asian CQ-resistant lines *Pf*Dd2 and *Pf*K1, as well as the ART-resistant line *Pf*Cam3.II and the ART- and piperaquine-resistant *Pf*RF7 line. The growth of all strains was inhibited by sub-micromolar concentrations of CHIR-124 but interestingly, an increased IC_{50} value (~3-fold less active) was observed against *Pf*Dd2 relative to the *Pf*NF54 wild-type strain (p-value=0.015, **Figure 1C**), whereas against the *Pf*K1 strain the IC_{50} value was indistinguishable (**Table 1** and **Supplementary Table S2**). This 3-fold increase in IC_{50} on the *Pf*Dd2 strain could indicate that the compound is marginally susceptible to multidrug resistance-mediated mechanisms as these are absent in the *Pf*K1 strain. Furthermore, CHIR-124 showed differences in dose-response curve profiles against the ART-resistant strains *Pf*Cam3.II and *Pf*RF7 relative to *Pf*NF54, where shallow or biphasic curves were observed (**Supplementary Figure S2** and **Supplementary Table S2**). These findings suggest that, while CHIR-124 remains active against drug-resistant strains, it is marginally cross-resistant with CQ and piperaquine. To further probe for cross-resistance, a pool of 45 DNA-barcoded resistant mutant lines[33,34] was exposed to $3\times IC_{50}$ concentrations of CHIR-124 (**Supplementary Table S4)**. No mutant strain outgrowth was observed after 14 days, suggesting negligible cross-resistance against known mutants (**Supplementary Figure S3**). We further investigated the ABS activity of CHIR-124 in rings versus trophozoites (**Figure 1D**) and observed minimal differences, suggesting that CHIR-124 targets processes of importance to both these stages.

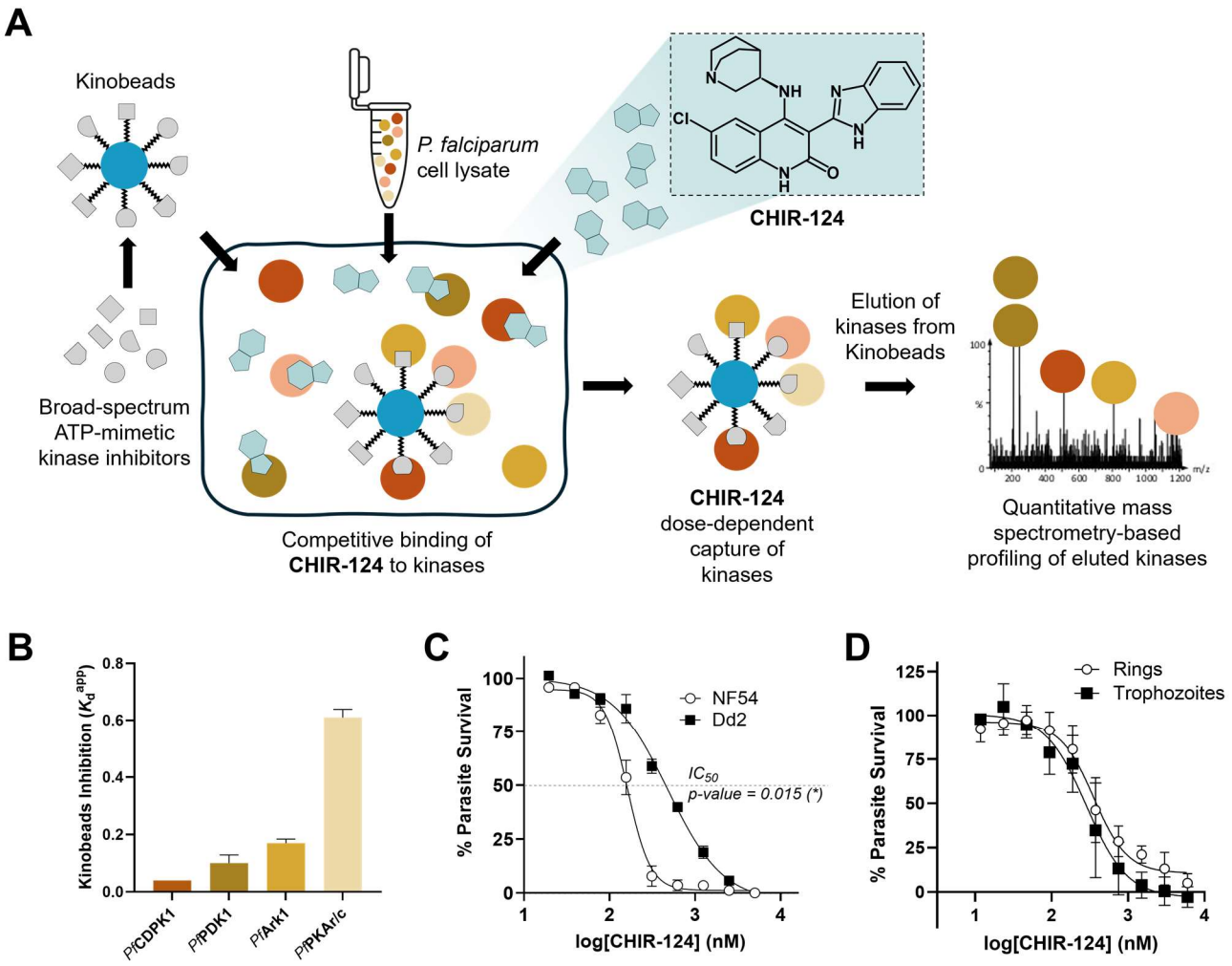

Figure 1. (**A**) The human kinase inhibitor CHIR-124 competitively binds to *Plasmodium* kinases captured on Kinobeads by broad spectrum kinase inhibitors immobilized on solid support beads. Eluted kinases are quantified in a dose-dependent manner via mass spectrometry. (**B**) Competitive binding for the top *Plasmodium* kinases with lowest apparent dissociation constant (K_d^{app}) in micromolar competed off Kinobeads by CHIR-124. Data and error bars represent the mean and SD from two independent experiments. (**C**) Dose-response profiles of CHIR-124 against the wild-type *Pf*NF54 strain vs the multidrug-resistant *Pf*Dd2 strain. Data are plotted as the mean with error bars representing the SEM where N,n=3,2. IC_{50} values are significantly different with $^*p<0.05$ via an unpaired two-tailed *t*-test. (**D**) Stage specificity of CHIR-124 in *Pf*NF54 showing dose response in rings and trophozoites. Data from rings vs trophozoites represent the mean ± SD from N,n=2,3-4 and are not significantly different.

Conditional Knockdown and Biochemical Studies Reveal Pf*Ark1 as a Target of CHIR-124*

We then further investigated each of the *Plasmodium* kinase targets competitively inhibited by CHIR-124 via the Kinobead studies in pursuit of the bona fide intracellular targets. The kinase with the lowest K_d^{app}, *Pf*CDPK1 (**Figure 1B**) is unlikely to contribute significantly to antiplasmodial activity based on previous studies showing that *Pf*CDPK1 is non-essential for ABS parasite survival.[35,36] Hence, we employed conditional knockdown (cKD) susceptibility assays with knockdowns of the remaining candidate kinases: *Pf*PDK1, *Pf*PKAc and *Pf*Ark1. We also included the cKDs of *Pf*Ark2 and the negative control *Pf*PI4K for comparison.

Two knockdown approaches were used. The first, for *Pf*PDK1, was based on tagging the endogenous *pfpdk1* gene with *gfp* fused to the *dd* sequence to generate the *Pf*PDK1-GFPDD protein in *Pf*NF54 WT parasites.[37] CHIR-124 was profiled against cultures under *Pf*PDK1-GFPDD-depleting conditions (–Shield-1) versus *Pf*PDK1-GFPDD-stabilizing conditions (+Shield-1). Similar CHIR-124 IC_{50} values were obtained regardless of *Pf*PDK1 protein expression, indicating negligible interaction between CHIR-124 and *Pf*PDK1 in whole cells (**Supplementary Table S5**).

170 **Table 1**. Life cycle activity, cross-resistance, stage specificity and biochemical inhibition data for CHIR-124.

	Assay	IC_{50} value ± SEM or SD[c] (μM)
Life cycle stage activity and cytotoxicity	Asexual blood stages (ABS, *Pf*NF54)	0.14 ± 0.03
	Immature gametocytes (*Pf*NF54)	3.1 ± 0.7
	Late-stage gametocytes (*Pf*NF54)	1.1 ± 0.2
	Liver stage (*P. berghei*)	0.31 ± 0.01
	Liver cytotoxicity, HepG2	4.3 ± 0.6 (SI: 13.9)[a]
ABS resistant strains	Drug-resistant *Pf*Dd2 strain	0.44 ± 0.05 (RI: 3.1)[b]
	Drug-resistant *Pf*K1 strain	0.14 ± 0.03 (RI: 1.0)[b]
Stage specificity	Ring stage *P. falciparum*	0.35 ± 0.03[c]
	Trophozoite *P. falciparum*	0.28 ± 0.03[c]
Biochemical kinase inhibition assays	*Pv*PI4K	>10
	*Pf*Ark1	0.38 ± 0.07[c]
	*Pf*Ark3	16 ± 2[c]
	*Pf*GSK3	0.20 ± 0.02[c]

171 [a] SI = Selectivity index (HepG2 IC_{50} value / Liver stage IC_{50} value). [b] RI = Resistance index (*Pf*Dd2 or *Pf*K1 IC_{50} value /
172 *Pf*NF54 IC_{50} value). [c] Data represent the mean ± SD.

173 The second cKD approach, used for *Pf*Ark1, *Pf*Ark2, *Pf*PKAc and *Pf*PI4K, relies on tetracycline transcriptional
174 regulator (TetR)-binding RNA aptamers, whereby binding to the aptamer is inhibited by increasing the concentration
175 of anhydrotetracycline (aTc) to allow downstream translation of mRNA and protein synthesis.[38] Normal levels of
176 kinase expression are achieved when ≥50 nM aTc is added to the culture prior to assay setup. The dose-response
177 activity of the cell line fully expressing the target (50 nM or 500 nM aTc) is then compared to that of the culture
178 lacking aTc in which expression of the kinase is repressed. Data showing the effect of the kinase knockdown on
179 untreated parasite viability is shown for *Pf*Ark1, *Pf*Ark2 and *Pf*PKAc in **Supplementary Figure S4**. Studies with
180 CHIR-124 treatment revealed that the *Pf*Ark1 cKD line where expression of *Pf*Ark1 is reduced using 1 nM aTc is
181 significantly more sensitive to CHIR-124 by 25-fold (*p*-value=0.011) relative to the line using 500 nM aTc that fully
182 expresses the kinase (left-shift of the dose-response curve, **Figure 2A**). Only minor shifts were observed with cKD
183 lines *Pf*PKAc and *Pf*Ark2 of 3.6-fold and 2.9-fold respectively, similar to those observed with the *Pf*PI4K negative
184 control line (**Supplementary Figure S4** and **Supplementary Table S5**), suggesting that these kinases do not play
185 significant roles in the antiplasmodial activity of CHIR-124.

186 CHIR-124 was also screened against available recombinant *Plasmodium* kinases (Luceome Biotechnologies) via
187 the competition binding KinaseSeeker™ assay.[39] In this assay format, each kinase is expressed in cell free lysate
188 without further purification. The assay measures displacement of a probe from the ATP binding site. Using this
189 approach, further validation of the interaction between CHIR-124 and *Pf*Ark1 was provided (*Pf*Ark1 IC_{50} 0.38 μM,
190 **Table 1**). In contrast, negligible inhibition of *Pf*Ark3 was observed (IC_{50} 16 μM), corroborating the fact that this
191 kinase was not identified as a putative target in the Kinobeads experiment. However, the most potent biochemical
192 activity for CHIR-124 against this kinase panel was seen for glycogen synthase kinase 3 beta (*Pf*GSK3β,
193 PF3D7_0312400) with an IC_{50} of 0.2 μM (**Table 1 and Supplementary Figure S5**). Notably, *Pf*GSK3β was also
194 identified in the Kinobeads experiments above, albeit with an IC_{50} of 3 μM (**Supplementary Figure S1**). Previous
195 work has shown that *Pf*GSK3β is not essential for growth in the ABS, but that inhibition leads to decreased growth
196 rates and growth defects.[40] Nevertheless, this does not exclude *Pf*GSK3 inhibition as a contributing mode of action
197 for CHIR-124.

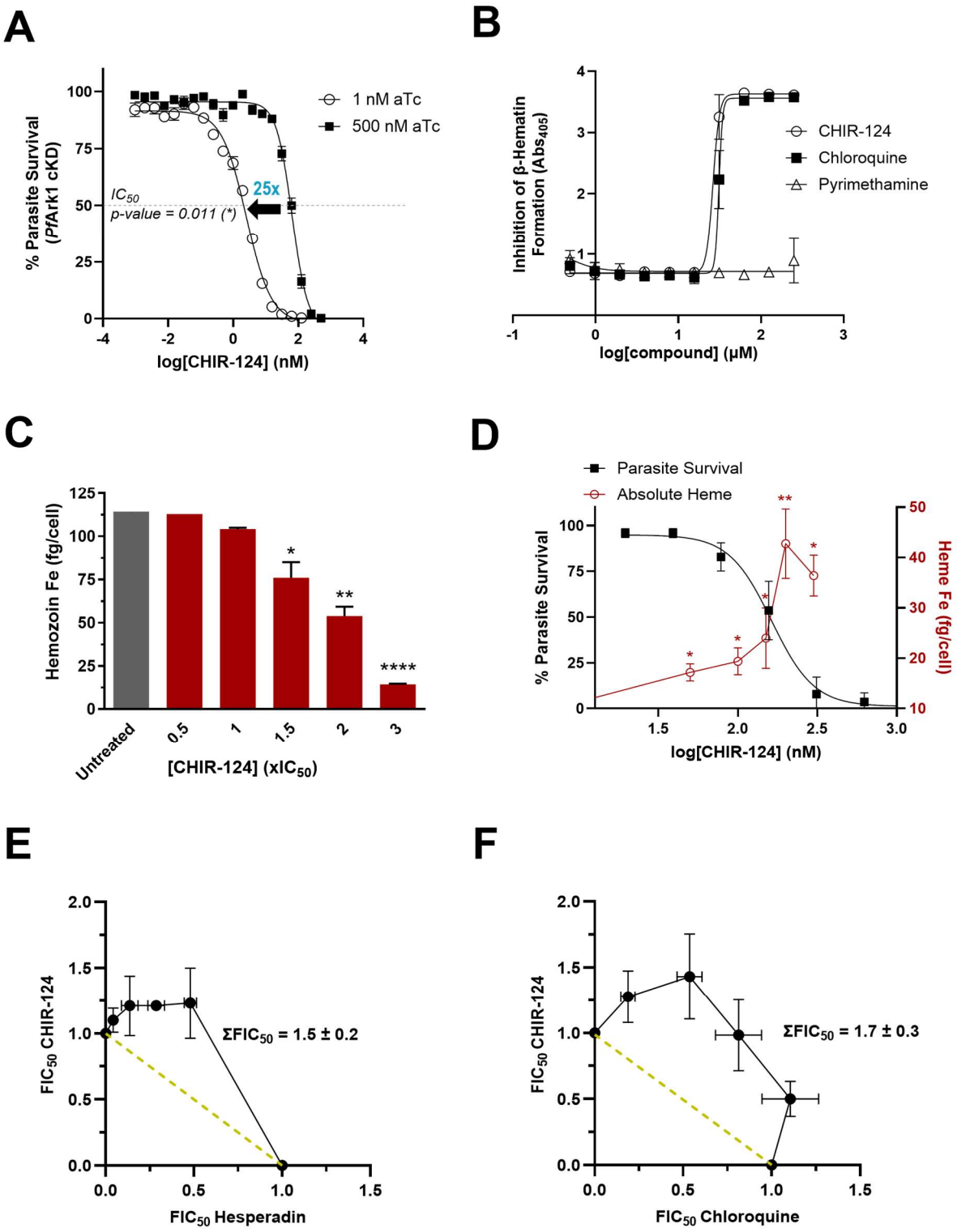

Figure 2. Genetic and phenotypic target exploration studies for CHIR-124. (**A**) The *Pf*Ark1 cKD line exposed to CHIR-124 under low (1 nM aTc) or high (500 nM aTc) conditions of protein expression. Data represents mean ± SEM where N,n=3,3. IC_{50} values are significantly different with **p*<0.05 via an unpaired two-tailed *t*-test. (**B**) β-hematin inhibition activity of CHIR-124 in the extracellular assay detergent-based biomimetic assay. Data represent mean ± SD (n=3). (**C**) Dose-dependent changes in the heme Fe levels from intracellularly-extracted fractions of hemozoin under CHIR-124 treatment (representative from N,n=3,3). Error bars represent ± SD for technical triplicates with an unpaired two-tailed *t*-test, where *p*-values are shown for **p*<0.05; ***p*<0.01; ****p*<0.001; *****p*<0.0001. (**D**) Parasite survival overlaid with the intracellularly-extracted fractions of free heme under CHIR-124 treatment. Significance determined as per panel (**C**). The correlation observed between the increasing heme levels with decreasing parasite survival indicates that hemozoin inhibition is a contributing mechanism of action of CHIR-124. Combination studies via fixed-ratio isobologram analysis showing an antagonistic effect on *Pf*NF54 ABS parasites for CHIR-124 with (**E**) the *Pf*Ark1 inhibitor hesperadin or (**F**) the hemozoin formation inhibitor chloroquine. Data represent mean fractional 50% inhibitory concentration (FIC_{50}) values ± SEM from three biological replicates each with 2–3 technical replicates. The mean sum of the FIC_{50} values (ΣFIC_{50}) ± SEM is also shown for each combination where ΣFIC_{50} >1.2 indicates antagonism.

CHIR-124 Inhibits the Formation of Intracellular Hemozoin

Given the structural features of CHIR-124 that resemble hemozoin formation inhibitors (i.e. planar heterocyclic rings with basic centers), the compound was also tested for its capacity to inhibit the formation of β-hematin (synthetic hemozoin) in an extracellular biomimetic detergent-mediated assay.[41] Intriguingly, the IC_{50} of CHIR-124 in this assay was comparable to that of the positive control antimalarial, chloroquine, a validated inhibitor of hemozoin formation[42] (**Figure 2B**), suggesting that CHIR-124 optimally interacts with free heme or crystalline β-hematin under biomimetic conditions to prevent further crystal growth. This finding prompted further whole-cell studies to better understand the effect that CHIR-124 has on the hemoglobin degradation process in the parasite. We therefore used an intracellular heme fractionation assay to probe the levels of hemoglobin, free heme and hemozoin in a dose-dependent manner when exposed to CHIR-124. While levels of hemoglobin uptake remained constant, an increase in free heme and decrease in hemozoin were observed over the range of 0.5–3×IC_{50} of CHIR-124 (**Figure 2C and Supplementary Figure S6**). Furthermore, the increase in free heme correlated with parasite growth inhibition (**Figure 2D**). This was a notable finding given that we have also shown that *Pf*Ark1 is an efficacious target for this compound in ABS parasites and, therefore, CHIR-124 represents one of the first phenotypically validated examples of a dual hemozoin formation inhibitor and kinase inhibitor.

An analogous hypothesis, that the molecular structure of CHIR-124 might facilitate chelation of the compound to metal ions such as Fe(II) and Fe(III) was also tested; however, a weak capacity of CHIR-124 to bind to physiologically-relevant metal ions suggested that disruption of metal homeostasis within the parasite was unlikely to contribute to its mode of action (**Supplementary Figure S7**). We additionally explored the phenotypic effect on the growth of *Pf*NF54 parasites by combining CHIR-124 with a primary *Pf*Ark1 or hemozoin formation inhibitor. Here, we employed the Ark1 inhibitor hesperadin[43,44] and the hemozoin formation inhibitor chloroquine.[42] Using this analysis, sum of the fractional IC_{50} (ΣFIC_{50}) values significantly greater than 1 indicate antagonism, those between approximately 0.8 to 1.4 indicate an additive effect, while those below 0.8 indicate synergy. Fixed ratio isobologram analysis revealed antagonistic interactions for both combinations, with ΣFIC_{50} values for CHIR-124 and hesperadin or CHIR-124 and chloroquine of 1.5 and 1.7, respectively (**Figures 2E** and **2F**). The antagonism observed suggests that CHIR-124 interacts within the same biological pathways as both hesperadin and chloroquine, which further supports its dual modes of action.[44]

CHIR-124 Affects Early Trophozoites and Prevents Proliferation from the Schizont Phase

Considering the polypharmacology displayed by CHIR-124, we sought to further probe the contribution of these different mechanistic pathways via morphological experiments. First, we treated tightly synchronized *Pf*NF54 parasites with 3×IC_{50} of CHIR-124 at four different stages (ring, late-ring, trophozoite or schizont) and monitored parasitemia and parasite morphology under drug pressure for every subsequent 12 h. CHIR-124 predominantly affected early trophozoites, whereby pyknotic parasites, unable to form healthy mature trophozoites, were visible within 12–24 h post-treatment (hpt) of rings and early trophozoites (**Figure 3A**). The nuclei in these parasites were also unable to divide to form schizonts, with the number of nuclei per cell (n) dramatically diminished in treated parasites compared to the untreated controls over 72 h (**Figure 3B**). This effect is especially striking at time points 24 h and 72 h (30–34 hpi and 78–82 hpi, respectively), which represent the schizont stages of untreated parasites. Analysis of the cell distribution via staining of parasite DNA with SYBR Green I further confirmed that CHIR-124-treated parasites were stalled in the early trophozoite phase (12 h) relative to the untreated population which showed a shift to the right (**Figure 3B**). However, CHIR-124 had little morphological effect on parasites already in the mature trophozoite phase, with hemozoin crystals visible (**Figure 3A**). These parasites were able to enter schizogony, albeit with fewer nuclei relative to the untreated control, and went on to form new rings.

To determine if the effect of CHIR-124 is reversible, we then performed similar experiments with a washout of CHIR-124 after 12 h and monitored the parasitemia in 12 h intervals (**Figure 3C**). Regardless of the starting point of treatment (measured at hpi), CHIR-124-treated parasites were unable to proliferate relative to the untreated control, despite the removal of drug pressure. These findings indicate that while CHIR-124 has the most significant morphological effect during the trophozoite phase due to its inhibition of hemozoin formation, it can arrest the growth of parasites treated during the schizont phase. In the latter, hemozoin formation is no longer relevant.[45] Instead this observation supports the inhibition of *Pf*Ark1, which is crucial for cell division and parasite proliferation. *Pf*Ark1 is one of the aurora-related (serine/threonine) kinases that regulates the separation of centrosomes by controlling bipolar microtubule spindle dynamics, chromosome segregation and cytokinesis.[46] Indeed, *Pf*Ark1 has been shown to be essential for erythrocytic schizogony and is localized to spindle pole bodies.[47,48] The abnormal

schizont morphology and lower yield nuclear division correlates with that reported for *Plasmodium* parasites treated with the *Pf*Ark1 inhibitor hesperadin.[43,44]

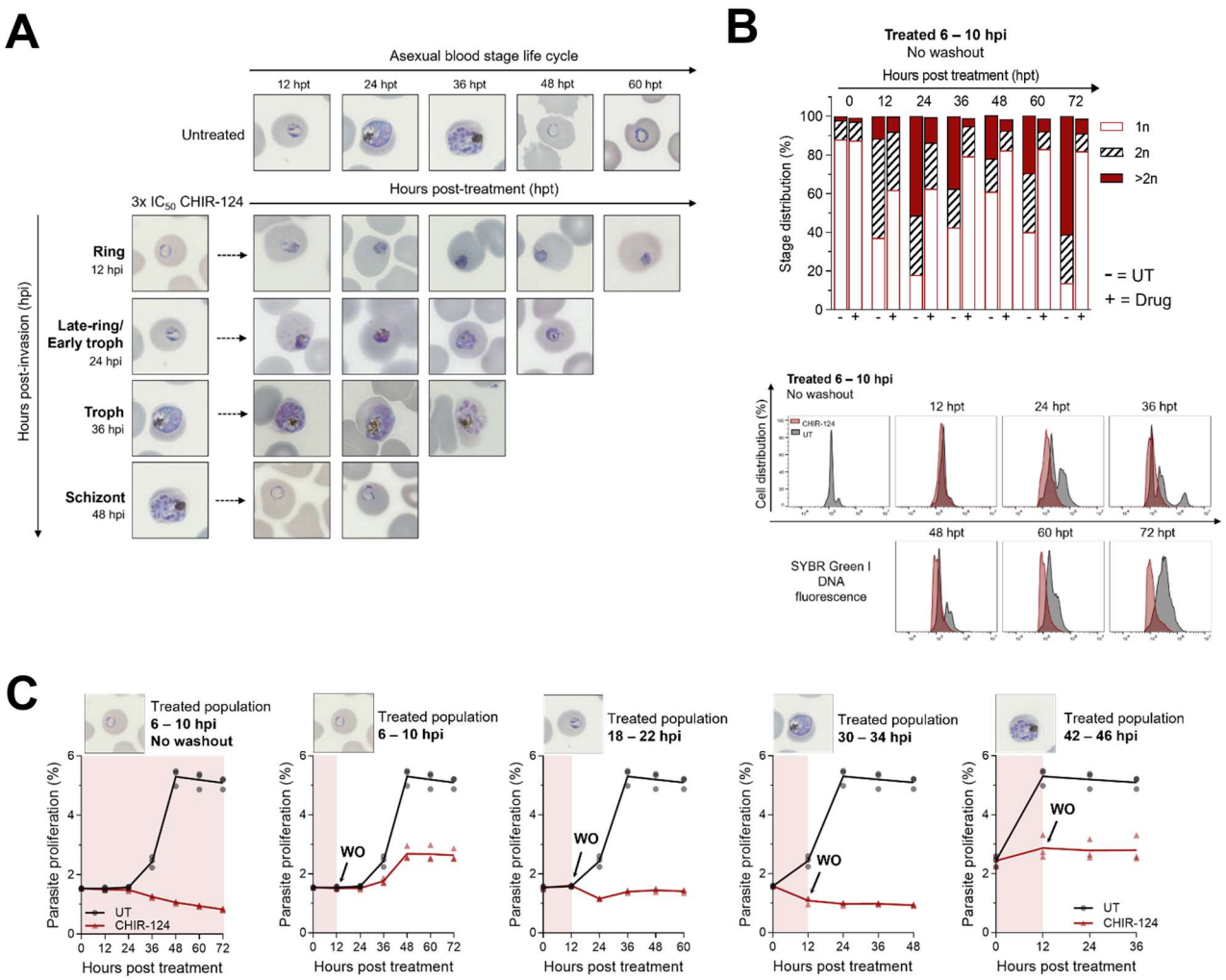

Figure 3. The morphology of ABS parasites treated with CHIR-124 at 3×IC_{50} vs untreated (UT) at various time-points indicated by hours post invasion (hpi) and samples at specific hours post treatment (hpt) where (**A**) shows images of Giemsa-stained blood smears, (**B**) shows the percentage stage distribution of the ABS parasites where schizonts are represented by number of nuclei per cell (n) as >2n, and (**C**) the growth of parasites when distinct synchronized populations are treated with CHIR-124 for 12 h.

The Risk of Resistance is Low for Parasites Treated with CHIR-124

One potential advantage of a compound that targets multiple pathways in the parasite is the lowered risk of resistance-conferring mutations arising simultaneously. To further probe the frequency of resistance for CHIR-124, 10^9 *Pf*Dd2 parasites were pressured with non-lethal doses of the compound (3×IC_{50}) until complete parasite clearance from the culture as visualized by microscopic examination from Giemsa-stained blood smears. The drug pressure was removed until parasites recrudesced (49 days after initializing the resistance selection). These parasites, however, showed no resistance phenotype (**Supplementary Figure S8**) and were therefore re-exposed to drug pressure at 5×IC_{50} until cleared, in attempt to select for higher-grade resistance. The culture was continued at 3×IC_{50} for 60 days. At this point no recrudescence had been observed, indicating that the minimum inoculum of resistance (MIR) under the *in vitro* experimental conditions for CHIR-124 is log(MIR) >9 against *Pf*Dd2. The lack of resistant mutants demonstrated a low level of risk for generating resistance against CHIR-124, and supported the observation of polypharmacology.[49]

Conclusion

Drug repositioning—using the scaffold of existing drugs as a starting point for antimalarial drug development—can significantly accelerate the optimization and deployment of effective treatments by reducing development time and costs. Herein we report the antiplasmodial activities of the human kinase inhibitor CHIR-124 and its potential to be repositioned as a dual-inhibitor for malaria. Dual-inhibitors exhibiting polypharmacology against *P. falciparum* represent a promising avenue for antimalarial drug development and offer a strategic advantage in overcoming the limitations of monotherapy. By inhibiting *Plasmodium* kinases as well as hemozoin formation, these compounds can disrupt key regulatory processes and detoxification mechanisms critical for parasite survival across multiple life cycle stages. Additionally, by simultaneously targeting multiple essential pathways within the parasite, dual-inhibitors have the potential to maintain activity against drug-resistant strains and reduce the likelihood of resistance, thereby improving treatment outcomes. Future work should focus on optimizing the selectivity, potency, and pharmacokinetic properties of CHIR-124 to facilitate its clinical translation. Ultimately, the development of such multitargeted and repositioned therapies through medicinal chemistry optimization could significantly advance malaria control and eradication efforts.

Supporting Information

The authors have cited additional references within the Supporting Information.[50-78]

Supplementary Figures S1 – S7, Supplementary Tables S1 – S4, protocols for Kinobeads screening, *Plasmodium falciparum* asexual blood stage parasitology methods, gametocyte and liver stage assays, cytotoxicity assays, stage specificity assays, conditional knockdown assays, β-hematin and hemozoin formation inhibition assays, metal chelation assays and biochemical assays.

Acknowledgements

South African Medical Research Council extramural unit funding to K.C. Medicines for Malaria Venture (MMV) African Challenge Grant RD-17-0047 awarded to D.C. and L-M.B and Project RD-19-0001 to L-M.B. Future Leaders−African Independent Research (FLAIR) Fellowship Programme, a partnership between the African Academy of Sciences and the Royal Society funded by the UK Government's Global Challenges Research to L.B.C. The National Institute of Allergy and Infectious Diseases of the National Institutes of Health to K.J.W. (R01AI143521), D.A.F. (R01AI185559) and K.C. (R01AI152092). E.A.W. is a member of the Malaria Drug Accelerator and is supported by a grant from the Bill and Melinda Gates Foundation (OPP1054480). The Department of Science and Innovation and the National Research Foundation South African Research Chair (SARChI) in Sustainable Malaria Control to L-M.B. (UID 84627). K.C. is the Neville Isdell Chair in African-centric Drug Discovery and Development and thanks Neville Isdell for generously funding the Chair.

Author Contributions

The manuscript was prepared by K.J.W. with contributions from other authors. β-hematin and metal chelation studies were conducted by J.G.W. Cell fractionation and combination studies were performed or analyzed by L.F.G. and K.J.W. Morphological studies were carried out by H.L. and L-M. B. Asexual blood stage parasite activity assays were performed by D.T., J.L.B. and D.A.F. Gametocyte assays were conducted by D.C., J.R, M.vdW and L-M.B. Liver-stage assays were done by M.L.S, J.L.S-N. and E.A.W. Kinobead experiments were conducted by S.G-D, M.J.L-M and F-J.G. Conditional knockdown experiments were performed and analyzed by L.C.G, C.F.P., J.C.N, C.S., S.W. and T.S.V. Barcoded mutant screening was conducted by G.G., R.C. and M.L. Resistance selections were carried out by K.J.W. The project was conceived and supervised by L.B.C and K.C.

Conflict of Interest: The authors declare no competing interests.

References

[1] S. Bhatt, D. J. Weiss, E. Cameron, D. Bisanzio, B. Mappin, U. Dalrymple, K. Battle, C. L. Moyes, A. Henry, P. A. Eckhoff, E. A. Wenger, O. Briet, M. A. Penny, T. A. Smith, A. Bennett, J. Yukich, T. P. Eisele, J. T. Griffin, C. A. Fergus, M. Lynch, F. Lindgren, J. M. Cohen, C. L. J. Murray, D. L. Smith, S. I. Hay, R. E. Cibulskis, P. W. Gething, The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015, *Nature* **2015**, *526*, 207-211.

[2] G. Siciliano, P. Alano, Enlightening the malaria parasite life cycle: bioluminescent *Plasmodium* in fundamental and applied research, *Front Microbiol* **2015**, *6*, 391.

[3] R. van Biljon, J. Niemand, R. van Wyk, K. Clark, B. Verlinden, C. Abrie, H. von Gruning, W. Smidt, A. Smit, J. Reader, H. Painter, M. Llinas, C. Doerig, L. M. Birkholtz, Inducing controlled cell cycle arrest and re-entry during asexual proliferation of *Plasmodium falciparum* malaria parasites, *Sci Rep* **2018**, *8*, 16581.

[4] D. S. Khoury, D. Cromer, S. E. Best, K. R. James, I. Sebina, A. Haque, M. P. Davenport, Reduced erythrocyte susceptibility and increased host clearance of young parasites slows *Plasmodium* growth in a murine model of severe malaria, *Sci Rep* **2015**, *5*, 9412.

[5] S. Yahiya, A. Rueda-Zubiaurre, M. J. Delves, M. J. Fuchter, J. Baum, The antimalarial screening landscape-looking beyond the asexual blood stage, *Curr Opin Chem Biol* **2019**, *50*, 1-9.

[6] J. N. Burrows, S. Duparc, W. E. Gutteridge, R. Hooft van Huijsduijnen, W. Kaszubska, F. Macintyre, S. Mazzuri, J. J. Mohrle, T. N. C. Wells, New developments in anti-malarial target candidate and product profiles, *Malar J* **2017**, *16*, 26.

[7] L. M. Birkholtz, P. Alano, D. Leroy, Transmission-blocking drugs for malaria elimination, *Trends Parasitol* **2022**, *38*, 390-403.

[8] L. M. Birkholtz, T. L. Coetzer, D. Mancama, D. Leroy, P. Alano, Discovering New Transmission-Blocking Antimalarial Compounds: Challenges and Opportunities, *Trends Parasitol* **2016**, *32*, 669-681.

[9] L. The, Malaria in 2022: a year of opportunity, *Lancet* **2022**, *399*, 1573.

[10] P. Venkatesan, WHO world malaria report 2024, *Lancet Microbe* **2025**, 101073.

[11] M. D. Conrad, P. J. Rosenthal, Antimalarial drug resistance in Africa: the calm before the storm?, *Lancet Infect Dis* **2019**, *19*, e338-e351.

[12] D. A. Fidock, P. J. Rosenthal, Artemisinin resistance in Africa: How urgent is the threat?, *Med (N Y)* **2021**, *2*, 1287-1288.

[13] J. L. Siqueira-Neto, K. J. Wicht, K. Chibale, J. N. Burrows, D. A. Fidock, E. A. Winzeler, Antimalarial drug discovery: progress and approaches, *Nat Rev Drug Discov* **2023**, *22*, 807-826.

[14] M. Duffey, B. Blasco, J. N. Burrows, T. N. C. Wells, D. A. Fidock, D. Leroy, Assessing risks of *Plasmodium falciparum* resistance to select next-generation antimalarials, *Trends Parasitol* **2021**, *37*, 709-721.

[15] S. Pushpakom, F. Iorio, P. A. Eyers, K. J. Escott, S. Hopper, A. Wells, A. Doig, T. Guilliams, J. Latimer, C. McNamee, A. Norris, P. Sanseau, D. Cavalla, M. Pirmohamed, Drug repurposing: progress, challenges and recommendations, *Nat Rev Drug Discov* **2019**, *18*, 41-58.

[16] S. Roy, S. Dhaneshwar, B. Bhasin, Drug Repurposing: An Emerging Tool for Drug Reuse, Recycling and Discovery, *Curr Drug Res Rev* **2021**, *13*, 101-119.

[17] A. Talevi, C. L. Bellera, Challenges and opportunities with drug repurposing: finding strategies to find alternative uses of therapeutics, *Expert Opin Drug Discov* **2020**, *15*, 397-401.

[18] M. Biddau, L. Sheiner, Targeting the apicoplast in malaria, *Biochem Soc Trans* **2019**, *47*, 973-983.

[19] M. Njoroge, N. M. Njuguna, P. Mutai, D. S. Ongarora, P. W. Smith, K. Chibale, Recent approaches to chemical discovery and development against malaria and the neglected tropical diseases human African trypanosomiasis and schistosomiasis, *Chem Rev* **2014**, *114*, 11138-11163.

[20] L. B. Arendse, J. M. Murithi, T. Qahash, C. F. A. Pasaje, L. C. Godoy, S. Dey, L. Gibhard, S. Ghidelli-Disse, G. Drewes, M. Bantscheff, M. J. Lafuente-Monasterio, S. Fienberg, L. Wambua, S. Gachuhi, D. Coertzen, M. van der Watt, J. Reader, A. S. Aswat, E. Erlank, N. Venter, N. Mittal, M. R. Luth, S. Ottilie, E. A. Winzeler, L. L. Koekemoer, L. M. Birkholtz, J. C. Niles, M. Llinas, D. A. Fidock, K. Chibale, The anticancer human mTOR inhibitor sapanisertib potently inhibits multiple *Plasmodium* kinases and life cycle stages, *Sci Transl Med* **2022**, *14*, eabo7219.

[21] H. D. Chien, A. Pantaleo, K. R. Kesely, P. Noomuna, K. S. Putt, T. A. Tuan, P. S. Low, F. M. Turrini, Imatinib augments standard malaria combination therapy without added toxicity, *J Exp Med* **2021**, *218*.

[22] L. B. Arendse, S. Wyllie, K. Chibale, I. H. Gilbert, *Plasmodium* Kinases as Potential Drug Targets for Malaria: Challenges and Opportunities, *ACS Infect Dis* **2021**, *7*, 518-534.

[23] T. G. Carvalho, B. Morahan, S. John von Freyend, P. Boeuf, G. Grau, J. Garcia-Bustos, C. Doerig, The ins and outs of phosphosignalling in *Plasmodium*: Parasite regulation and host cell manipulation, *Mol Biochem Parasitol* **2016**, *208*, 2-15.

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

[25] P. Sinxadi, C. Donini, H. Johnstone, G. Langdon, L. Wiesner, E. Allen, S. Duparc, S. Chalon, J. S. McCarthy, U. Lorch, K. Chibale, J. Mohrle, K. I. Barnes, Safety, Tolerability, Pharmacokinetics, and Antimalarial Activity of the Novel *Plasmodium* Phosphatidylinositol 4-Kinase Inhibitor MMV390048 in Healthy Volunteers, *Antimicrob Agents Chemother* **2020**, *64*.

[26] X. C. Ding, D. Ubben, T. N. Wells, A framework for assessing the risk of resistance for anti-malarials in development, *Malar J* **2012**, *11*, 292.

[27] M. Patil, N. Pabla, Z. Dong, Checkpoint kinase 1 in DNA damage response and cell cycle regulation, *Cell Mol Life Sci* **2013**, *70*, 4009-4021.

[28] C. S. Sorensen, R. G. Syljuasen, J. Falck, T. Schroeder, L. Ronnstrand, K. K. Khanna, B. B. Zhou, J. Bartek, J. Lukas, Chk1 regulates the S phase checkpoint by coupling the physiological turnover and ionizing radiation-induced accelerated proteolysis of Cdc25A, *Cancer Cell* **2003**, *3*, 247-258.

[29] A. N. Tse, K. G. Rendahl, T. Sheikh, H. Cheema, K. Aardalen, M. Embry, S. Ma, E. J. Moler, Z. J. Ni, D. E. Lopes de Menezes, B. Hibner, T. G. Gesner, G. K. Schwartz, CHIR-124, a novel potent inhibitor of Chk1, potentiates the cytotoxicity of topoisomerase I poisons in vitro and in vivo, *Clin Cancer Res* **2007**, *13*, 591-602.

[30] L. Dittus, T. Werner, M. Muelbaier, M. Bantscheff, Differential Kinobeads Profiling for Target Identification of Irreversible Kinase Inhibitors, *ACS Chem Biol* **2017**, *12*, 2515-2521.

[31] G. Medard, F. Pachl, B. Ruprecht, S. Klaeger, S. Heinzlmeir, D. Helm, H. Qiao, X. Ku, M. Wilhelm, T. Kuehne, Z. Wu, A. Dittmann, C. Hopf, K. Kramer, B. Kuster, Optimized chemical proteomics assay for kinase inhibitor profiling, *J Proteome Res* **2015**, *14*, 1574-1586.

[32] R. van Biljon, R. van Wyk, H. J. Painter, L. Orchard, J. Reader, J. Niemand, M. Llinas, L. M. Birkholtz, Hierarchical transcriptional control regulates *Plasmodium falciparum* sexual differentiation, *BMC Genomics* **2019**, *20*, 920.

[33] M. Carrasquilla, N. F. Drammeh, M. Rawat, T. Sanderson, Z. Zenonos, J. C. Rayner, M. C. S. Lee, Barcoding Genetically Distinct *Plasmodium falciparum* Strains for Comparative Assessment of Fitness and Antimalarial Drug Resistance, *mBio* **2022**, *13*, e0093722.
[34] D. Mambwe, D. Coertzen, M. Leshabane, M. Mulubwa, M. Njoroge, L. Gibhard, G. Girling, K. J. Wicht, M. C. S. Lee, S. Wittlin, D. R. M. Moreira, L. M. Birkholtz, K. Chibale, hERG, *Plasmodium* Life Cycle, and Cross Resistance Profiling of New Azabenzimidazole Analogues of Astemizole, *ACS Med Chem Lett* **2024**, *15*, 463-469.
[35] A. Bansal, K. K. Ojo, J. Mu, D. J. Maly, W. C. Van Voorhis, L. H. Miller, Reduced Activity of Mutant Calcium-Dependent Protein Kinase 1 Is Compensated in *Plasmodium falciparum* through the Action of Protein Kinase G, *mBio* **2016**, *7*.
[36] M. Vanaerschot, J. M. Murithi, C. F. A. Pasaje, S. Ghidelli-Disse, L. Dwomoh, M. Bird, N. Spottiswoode, N. Mittal, L. B. Arendse, E. S. Owen, K. J. Wicht, G. Siciliano, M. Bosche, T. Yeo, T. R. S. Kumar, S. Mok, E. F. Carpenter, M. J. Giddins, O. Sanz, S. Ottilie, P. Alano, K. Chibale, M. Llinas, A. C. Uhlemann, M. Delves, A. B. Tobin, C. Doerig, E. A. Winzeler, M. C. S. Lee, J. C. Niles, D. A. Fidock, Inhibition of Resistance-Refractory *P. falciparum* Kinase PKG Delivers Prophylactic, Blood Stage, and Transmission-Blocking Antiplasmodial Activity, *Cell Chem Biol* **2020**, *27*, 806-816 e808.
[37] E. Hitz, N. Wiedemar, A. Passecker, B. A. S. Graca, C. Scheurer, S. Wittlin, N. M. B. Brancucci, I. Vakonakis, P. Maser, T. S. Voss, The 3-phosphoinositide-dependent protein kinase 1 is an essential upstream activator of protein kinase A in malaria parasites, *PLoS Biol* **2021**, *19*, e3001483.
[38] S. J. Goldfless, J. C. Wagner, J. C. Niles, Versatile control of *Plasmodium falciparum* gene expression with an inducible protein-RNA interaction, *Nat Commun* **2014**, *5*, 5329.
[39] B. W. Jester, K. J. Cox, A. Gaj, C. D. Shomin, J. R. Porter, I. Ghosh, A coiled-coil enabled split-luciferase three-hybrid system: applied toward profiling inhibitors of protein kinases, *J Am Chem Soc* **2010**, *132*, 11727-11735.
[40] A. Alder, L. Wilcke, E. Pietsch, H. von Thien, S. Pazicky, C. Low, P. Mesen-Ramirez, A. Bachmann, P. C. Burda, C. Kunick, H. Sondermann, D. Wilson, T. W. Gilberger, Functional inactivation of *Plasmodium falciparum* glycogen synthase kinase GSK3 modulates erythrocyte invasion and blocks gametocyte maturation, *J Biol Chem* **2022**, *298*, 102360.
[41] M. D. Carter, V. V. Phelan, R. D. Sandlin, B. O. Bachmann, D. W. Wright, Lipophilic mediated assays for beta-hematin inhibitors, *Comb Chem High Throughput Screen* **2010**, *13*, 285-292.
[42] J. M. Combrinck, K. Y. Fong, L. Gibhard, P. J. Smith, D. W. Wright, T. J. Egan, Optimization of a multi-well colorimetric assay to determine haem species in *Plasmodium falciparum* in the presence of anti-malarials, *Malar J* **2015**, *14*, 253.
[43] B. J. Morahan, C. Abrie, K. Al-Hasani, M. B. Batty, V. Corey, A. N. Cowell, J. Niemand, E. A. Winzeler, L. M. Birkholtz, C. Doerig, J. F. Garcia-Bustos, Human Aurora kinase inhibitor Hesperadin reveals epistatic interaction between *Plasmodium falciparum* PfArk1 and PfNek1 kinases, *Commun Biol* **2020**, *3*, 701.
[44] H. Langeveld, K. Maepa, M. Maree, J. L. Thibaud, N. Salomane, R. Bridgwater, M. T. Famodimu, L. C. Godoy, C. F. A. Pasaje, N. Boonyalai, M. L. de Souza, J. Fong, T. Rabie, R. P. Theart, S. Ghidelli-Disse, J. C. Niles, M. C. S. Lee, E. A. Winzeler, M. J. Delves, K. Chibale, K. J. Wicht, L. B. Coulson, L.-M. Birkholtz, Targeting Aurora kinases as essential cell cycle regulators to deliver multi-stage antimalarials against *Plasmodium falciparum*, *bioRxiv* **2025**, 2025.2007.2010.663886.
[45] L. F. Garnie, T. J. Egan, K. J. Wicht, Heme Detoxification in the Malaria Parasite *Plasmodium falciparum*: A Time-Dependent Basal-Level Analysis, *bioRxiv* **2025**.
[46] T. G. Carvalho, C. Doerig, L. Reininger, Nima- and Aurora-related kinases of malaria parasites, *Biochim Biophys Acta* **2013**, *1834*, 1336-1345.
[47] L. Reininger, J. M. Wilkes, H. Bourgade, D. Miranda-Saavedra, C. Doerig, An essential Aurora-related kinase transiently associates with spindle pole bodies during *Plasmodium falciparum* erythrocytic schizogony, *Mol Microbiol* **2011**, *79*, 205-221.
[48] M. Wyss, B. T. Thommen, J. Kofler, E. Carrington, N. M. B. Brancucci, T. S. Voss, The three *Plasmodium falciparum* Aurora-related kinases display distinct temporal and spatial associations with mitotic structures in asexual blood stage parasites and gametocytes, *mSphere* **2024**, *9*, e0046524.
[49] B. K. Verlinden, A. Louw, L. M. Birkholtz, Resisting resistance: is there a solution for malaria?, *Expert Opin Drug Discov* **2016**, *11*, 395-406.
[50] L. B. Arendse, J. M. Murithi, T. Qahash, C. F. A. Pasaje, L. C. Godoy, S. Dey, L. Gibhard, S. Ghidelli-Disse, G. Drewes, M. Bantscheff, M. J. Lafuente-Monasterio, S. Fienberg, L. Wambua, S. Gachuhi, D. Coertzen, M. van der Watt, J. Reader, A. S. Aswat, E. Erlank, N. Venter, N. Mittal, M. R. Luth, S. Ottilie, E. A. Winzeler, L. L. Koekemoer, L. M. Birkholtz, J. C. Niles, M. Llinas, D. A. Fidock, K. Chibale, The anticancer human mTOR inhibitor sapanisertib potently inhibits multiple *Plasmodium* kinases and life cycle stages, *Sci Transl Med* **2022**, *14*, eabo7219.
[51] E. Hitz, N. Wiedemar, A. Passecker, B. A. S. Graca, C. Scheurer, S. Wittlin, N. M. B. Brancucci, I. Vakonakis, P. Maser, T. S. Voss, The 3-phosphoinositide-dependent protein kinase 1 is an essential upstream activator of protein kinase A in malaria parasites, *PLoS Biol* **2021**, *19*, e3001483.
[52] S. J. Goldfless, J. C. Wagner, J. C. Niles, Versatile control of *Plasmodium falciparum* gene expression with an inducible protein-RNA interaction, *Nat Commun* **2014**, *5*, 5329.
[53] A. C. Balestra, K. Koussis, N. Klages, S. A. Howell, H. R. Flynn, M. Bantscheff, C. Pasquarello, A. J. Perrin, L. Brusini, P. Arboit, O. Sanz, L. P. Castano, C. Withers-Martinez, A. Hainard, S. Ghidelli-Disse, A. P. Snijders, D. A. Baker, M. J. Blackman, M. Brochet, Ca(2+) signals critical for egress and gametogenesis in malaria parasites depend on a multipass membrane protein that interacts with PKG, *Sci Adv* **2021**, *7*.
[54] M. Bantscheff, C. Hopf, M. M. Savitski, A. Dittmann, P. Grandi, A. M. Michon, J. Schlegl, Y. Abraham, I. Becher, G. Bergamini, M. Boesche, M. Delling, B. Dumpelfeld, D. Eberhard, C. Huthmacher, T. Mathieson, D. Poeckel, V. Reader, K. Strunk, G. Sweetman, U. Kruse, G. Neubauer, N. G. Ramsden, G. Drewes, Chemoproteomics profiling of HDAC inhibitors reveals selective targeting of HDAC complexes, *Nat Biotechnol* **2011**, *29*, 255-265.
[55] T. Werner, G. Sweetman, M. F. Savitski, T. Mathieson, M. Bantscheff, M. M. Savitski, Ion coalescence of neutron encoded TMT 10-plex reporter ions, *Anal Chem* **2014**, *86*, 3594-3601.
[56] S. Moggridge, P. H. Sorensen, G. B. Morin, C. S. Hughes, Extending the Compatibility of the SP3 Paramagnetic Bead Processing Approach for Proteomics, *J Proteome Res* **2018**, *17*, 1730-1740.
[57] S. Sridharan, N. Kurzawa, T. Werner, I. Gunthner, D. Helm, W. Huber, M. Bantscheff, M. M. Savitski, Proteome-wide solubility and thermal stability profiling reveals distinct regulatory roles for ATP, *Nat Commun* **2019**, *10*, 1155.
[58] W. Trager, J. B. Jensen, Human malaria parasites in continuous culture, *Science* **1976**, *193*, 673-675.
[59] M. T. Makler, J. M. Ries, J. A. Williams, J. E. Bancroft, R. C. Piper, B. L. Gibbins, D. J. Hinrichs, Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity, *Am J Trop Med Hyg* **1993**, *48*, 739-741.
[60] C. Le Manach, C. Scheurer, S. Sax, S. Schleiferbock, D. G. Cabrera, Y. Younis, T. Paquet, L. Street, P. Smith, X. C. Ding, D. Waterson, M. J. Witty, D. Leroy, K. Chibale, S. Wittlin, Fast in vitro methods to determine the speed of action and the stage-specificity of anti-malarials in *Plasmodium falciparum*, *Malar J* **2013**, *12*, 424.
[61] J. Reader, M. E. van der Watt, L. M. Birkholtz, Streamlined and Robust Stage-Specific Profiling of Gametocytocidal Compounds Against *Plasmodium falciparum*, *Front Cell Infect Microbiol* **2022**, *12*, 926460.

[62] A. K. Pathak, J. C. Shiau, B. Franke-Fayard, L. M. Shollenberger, D. A. Harn, D. E. Kyle, C. C. Murdock, Streamlining sporozoite isolation from mosquitoes by leveraging the dynamics of migration to the salivary glands, *Malar J* **2022**, *21*, 264.
[63] S. Yalaoui, S. Zougbede, S. Charrin, O. Silvie, C. Arduise, K. Farhati, C. Boucheix, D. Mazier, E. Rubinstein, P. Froissard, Hepatocyte permissiveness to *Plasmodium* infection is conveyed by a short and structurally conserved region of the CD81 large extracellular domain, *PLoS Pathog* **2008**, *4*, e1000010.
[64] J. Swann, V. Corey, C. A. Scherer, N. Kato, E. Comer, M. Maetani, Y. Antonova-Koch, C. Reimer, K. Gagaring, M. Ibanez, D. Plouffe, A. M. Zeeman, C. H. Kocken, C. W. McNamara, S. L. Schreiber, B. Campo, E. A. Winzeler, S. Meister, High-Throughput Luciferase-Based Assay for the Discovery of Therapeutics That Prevent Malaria, *ACS Infect Dis* **2016**, *2*, 281-293.
[65] C. Snyder, J. Chollet, J. Santo-Tomas, C. Scheurer, S. Wittlin, In vitro and in vivo interaction of synthetic peroxide RBx11160 (OZ277) with piperaquine in *Plasmodium* models, *Exp Parasitol* **2007**, *115*, 296-300.
[66] S. M. Ganesan, A. Falla, S. J. Goldfless, A. S. Nasamu, J. C. Niles, Synthetic RNA-protein modules integrated with native translation mechanisms to control gene expression in malaria parasites, *Nat Commun* **2016**, *7*, 10727.
[67] A. S. Nasamu, A. Falla, C. F. A. Pasaje, B. A. Wall, J. C. Wagner, S. M. Ganesan, S. J. Goldfless, J. C. Niles, An integrated platform for genome engineering and gene expression perturbation in *Plasmodium falciparum*, *Sci Rep* **2021**, *11*, 342.
[68] K. Deitsch, C. Driskill, T. Wellems, Transformation of malaria parasites by the spontaneous uptake and expression of DNA from human erythrocytes, *Nucleic Acids Res* **2001**, *29*, 850-853.
[69] M. Carrasquilla, N. F. Drammeh, M. Rawat, T. Sanderson, Z. Zenonos, J. C. Rayner, M. C. S. Lee, Barcoding Genetically Distinct *Plasmodium falciparum* Strains for Comparative Assessment of Fitness and Antimalarial Drug Resistance, *mBio* **2022**, *13*, e0093722.
[70] D. Mambwe, D. Coertzen, M. Leshabane, M. Mulubwa, M. Njoroge, L. Gibhard, G. Girling, K. J. Wicht, M. C. S. Lee, S. Wittlin, D. R. M. Moreira, L. M. Birkholtz, K. Chibale, hERG, *Plasmodium* Life Cycle, and Cross Resistance Profiling of New Azabenzimidazole Analogues of Astemizole, *ACS Med Chem Lett* **2024**, *15*, 463-469.
[71] Q. L. Fivelman, I. S. Adagu, D. C. Warhurst, Modified fixed-ratio isobologram method for studying in vitro interactions between atovaquone and proguanil or dihydroartemisinin against drug-resistant strains of *Plasmodium falciparum*, *Antimicrob Agents Chemother* **2004**, *48*, 4097-4102.
[72] R. van Biljon, J. Niemand, R. van Wyk, K. Clark, B. Verlinden, C. Abrie, H. von Gruning, W. Smidt, A. Smit, J. Reader, H. Painter, M. Llinas, C. Doerig, L. M. Birkholtz, Inducing controlled cell cycle arrest and re-entry during asexual proliferation of *Plasmodium falciparum* malaria parasites, *Sci Rep* **2018**, *8*, 16581.
[73] R. D. Sandlin, M. D. Carter, P. J. Lee, J. M. Auschwitz, S. E. Leed, J. D. Johnson, D. W. Wright, Use of the NP-40 detergent-mediated assay in discovery of inhibitors of beta-hematin crystallization, *Antimicrob Agents Chemother* **2011**, *55*, 3363-3369.
[74] K. K. Ncokazi, T. J. Egan, A colorimetric high-throughput beta-hematin inhibition screening assay for use in the search for antimalarial compounds, *Anal Biochem* **2005**, *338*, 306-319.
[75] J. M. Combrinck, K. Y. Fong, L. Gibhard, P. J. Smith, D. W. Wright, T. J. Egan, Optimization of a multi-well colorimetric assay to determine haem species in *Plasmodium falciparum* in the presence of anti-malarials, *Malar J* **2015**, *14*, 253.
[76] J. G. Woodland, R. Hunter, P. J. Smith, T. J. Egan, Shining new light on ancient drugs: preparation and subcellular localisation of novel fluorescent analogues of Cinchona alkaloids in intraerythrocytic *Plasmodium falciparum*, *Org Biomol Chem* **2017**, *15*, 589-597.
[77] B. W. Jester, K. J. Cox, A. Gaj, C. D. Shomin, J. R. Porter, I. Ghosh, A coiled-coil enabled split-luciferase three-hybrid system: applied toward profiling inhibitors of protein kinases, *J Am Chem Soc* **2010**, *132*, 11727-11735.
[78] B. W. Jester, A. Gaj, C. D. Shomin, K. J. Cox, I. Ghosh, Testing the promiscuity of commercial kinase inhibitors against the AGC kinase group using a split-luciferase screen, *J Med Chem* **2012**, *55*, 1526-1537.