Detection of the \textit{in vitro} modulation of \textit{Plasmodium falciparum} Arf1 by Sec7 and ArfGAP domains using a colorimetric plate-based assay

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The regulation of human Arf1 GTPase activity by ArfGEFs that stimulate GDP/GTP exchange and ArfGAPs that mediate GTP hydrolysis has attracted attention for the discovery of Arf1 inhibitors as potential anti-cancer agents. The malaria parasite \textit{Plasmodium falciparum} encodes a Sec7 domain-containing protein - presumably an ArfGEF - and two putative ArfGAPs, as well as an Arf1 homologue (\textit{Pf}Arf1) that is essential for blood-stage parasite viability. However, ArfGEF and ArfGAP-mediated activation/deactivation of \textit{Pf}Arf1 has not been demonstrated. In this study, we established an \textit{in vitro} colorimetric microtiter plate-based assay to detect the activation status of truncated human and \textit{P}. \textit{falciparum} Arf1 and used it to demonstrate the activation of both proteins by the Sec7 domain of ARNO, their deactivation by the GAP domain of human ArfGAP1 and the inhibition of the respective reactions by the compounds SecinH3 and QS11. In addition, we found that the GAP domains of both \textit{P}. \textit{falciparum} ArfGAPs have activities equivalent to that of human ArfGAP1, but are insensitive to QS11. Library screening identified a novel inhibitor which selectively inhibits one of the \textit{P. falciparum} GAP domains (IC\textsubscript{50} 4.7 µM), suggesting that the assay format is suitable for screening compound collections for inhibitors of Arf1 regulatory proteins.

ADP-ribosylation factor (Arf) GTPases are central regulators of protein trafficking in eukaryotic cells. There are six Arf isoforms, divided into three classes based on sequence homology, of which the most widely studied are Arf1 (Class I) and Arf6 (Class III). Respectively, they principally mediate trafficking in the secretory (Arf1) and endocytic (Arf6) pathways, with additional roles for Arf6 in actin cytoskeleton dynamics\textsuperscript{1-3}. Arf1 is the focus of this study and initiates vesicle formation in the Golgi apparatus by activating lipid modifying enzymes and recruiting coatomer complex I (COPI) coat proteins. The COPI vesicles are responsible for retrograde transport of cargo and trafficking proteins to earlier Golgi compartments and the endoplasmic reticulum\textsuperscript{4}. In addition, Arf1 recruits clathrin adaptor proteins (AP1, AP3 and AP4) and Golgi-localized \textgamma-ear-containing ARF-binding (GGA) proteins to the trans-Golgi network, where they are involved in trapping cargo proteins and the formation of vesicles that deliver secretory proteins to endosomes\textsuperscript{5}.

Presumably, the delivery of newly synthesised secretory proteins to their correct locations places a heavy burden on Arf1 activity in rapidly growing cells. Indeed, Arf1 is upregulated in cancer cell types and plays a role in cancer metastasis phenotypes e.g. cell detachment, migration and invasion, and may additionally be involved in tumour-promoting cell signalling pathways e.g. the phosphatidylinositol 3-kinase (PI3K) and mitogen-activate protein kinase (MAPK) pathways\textsuperscript{6-9}. Moreover, Arf1 inhibitors inhibit cancer cell viability, proliferation and metastatic characteristics\textsuperscript{10} and tumour growth in mouse models\textsuperscript{11-13}. Like other small GTPases, Arf1 undergoes a cycle of activation and deactivation that is determined by its nucleotide binding status. Exchanging GDP for GTP

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activates Arf1 through a pronounced conformational change which exposes a myristoylated N-terminal amphipathic α-helix, resulting in membrane association, and enhances effector protein binding. Conversely, hydrolysis of the terminal phosphate of the bound GTP to form GDP deactivates Arf1, returning it to a cytoplasmic pool. Due to the low intrinsic nucleotide exchange and hydrolysis activity of Arf1, Arf1 activation is stimulated by a family of guanine nucleotide exchange factors (GEFs) containing a characteristic Sec7 domain\(^\text{14}\), while deactivation is promoted by GTPase activating proteins (GAPs) containing GAP domains\(^\text{13,15}\). The development of Arf1 inhibitors has focused on compounds that disrupt GEF-mediated nucleotide exchange (although the detailed mechanisms may differ) and includes \textit{inter alia} brefeldin A (BFA) and its analogues, Golgicide A, AMF-26, LM11, Exo2 and SecinH3\(^\text{11,16-20}\). However, Arf GAP inhibitors – QS11 and its derivatives – have been described and reported to inhibit the migration of breast cancer cells\(^\text{21,22}\).

The genome of the most prevalent and virulent of the malaria parasite species, Plasmodium falciparum, contains six sequences that have been annotated as encoding putative Arf or Arf-like proteins (www.plasmodb.org). One such sequence encodes an Arf1 homologue (\textit{Pf}Arf1) that has a very high amino acid sequence conservation (76% identity, 89% similarity) compared to human Arf1. Originally identified by probing a \textit{P. falciparum} genomic library and PCR from \textit{P. falciparum} cDNA\(^\text{23-26}\), the recombinant protein was shown to bind GTP, have ADP-ribosyltransferase and phospholipase D stimulating activity in addition to low intrinsic GTPase activity, all features of Arf GT-Pases\(^\text{14,23}\). It is also capable of stimulating \textit{P. falciparum} phosphatidylinositol 4-phosphate-5-kinase (PIP\textsubscript{5}K), which is an established role of mammalian Arf1 in the regulation of phosphorylated phosphatidylinositol levels and, consequently, membrane trafficking, signalling and cytoskeleton dynamics\(^\text{46}\). In blood-stage parasites, \textit{Pf}Arf1 fused to GFP was found to co-localise with the Golgi marker GRASP\(^\text{27}\), while the canonical Arf1 activation inhibitor BFA causes a disruption in Golgi architecture and trafficking of secretory proteins\(^\text{28-32}\). Taken together, these studies suggest that \textit{Pf}Arf1 mimics the key role of mammalian Arf1 in secretory traffic through the Golgi apparatus. As would be expected based on sequence conservation, the crystal structure of GDP-bound \textit{Pf}Arf1 is very similar to that of human Arf1, with subtle differences in the Switch I and II domains that could affect binding of GEFs and GAPs\(^\text{33}\). However, direct demonstration of GEF-mediated nucleotide exchange and GAP-mediated GTP hydrolysis by \textit{Pf}Arf1 has not been reported.

Interestingly, unlike mammalian cells where the Arf GEF and GAP families contain up to 15 and 27 members respectively\(^\text{14}\), the \textit{P. falciparum} genome encodes two putative ArfGAP proteins and a single Sec7 domain-containing putative ArfGEF, responsible for the BFA sensitivity of malaria parasites\(^\text{34,35}\). The crystal structure of the catalytic GAP domain of one of the GAP isoforms (designated \textit{Pf}ArfGAP1 in this study) has been determined and shows an overall similarity of tertiary structure compared to mammalian GAP domains\(^\text{36}\). However, unlike the highly conserved \textit{Pf}Arf1, there is a greater divergence of amino acid sequence homology compared to human ArfGAP1 (39% identity and 52% similarity) and differences in the amino acid residues predicted to interact with Arf1\(^\text{36}\). In this study, using human recombinant proteins as a model, we developed a novel microtiter plate-based assay to detect Arf1 activation (GTP vs. GDP-bound) status and modulation of it by an ArfGEF (ARNO) Sec7 domain and Arf GAP (ArfGAP1) GAP domain. We used the assay to demonstrate and compare the Arf1 GAP activities of the GAP domains of the two putative \textit{P. falciparum} GAPS, as well as demonstrate ARNO-stimulated nucleotide exchange by \textit{Pf}Arf1. Given the interest in Arf1 as a drug target, a further motivation for developing the assay was to introduce an assay format compatible with the screening of compound libraries for Arf1 activity modulators, explored here by detecting the differential inhibition of ARNO and GAP-mediated Arf1 activation/deactivation using standard inhibitors, as well as the identification of a novel, selective \textit{Pf}Arf1 GAP inhibitor.

**Results**

**A colorimetric plate-based GST-GGA3 binding assay discriminates between GTP- and GDP-bound Arf1.** The phenomenon that Arf1only binds to the coat protein GGA3 (via the GAT domain of the latter) when it is in its active GTP-bound vs. inactive GDP-bound conformation has been widely employed as an experimental tool to detect Arf1 activation status in cultured cells using pull-down assays. Typically, glutathione beads coated with a fusion protein consisting of glutathione-S-transferase (GST) and the GAT domain of GGA3 (GST-GGA3\(^\text{GAT}\)) are incubated with cell lysates and bead-bound (active) vs. total Arf1 levels determined by western blotting\(^\text{37}\). To determine if the selective binding of GST-GGA3 GAT to Arf1-GTP could be further exploited to determine the activation status of purified recombinant Arf1 proteins in a microtiter plate format, we conceptualised an assay procedure (Fig. S1) in which Arf1, expressed and purified as a truncated histidine-tagged protein (Fig. S2), is immobilised on nickel-NTA coated 96-well plates, followed by incubation with purified GST-GGA3\(^\text{GAT}\). The extent of GST-GGA3\(^\text{GAT}\) binding to the plate may be readily determined by the addition of a colorimetric GST enzyme substrate, and should correlate with the level of GTP-bound Arf1. Assessing the viability of this approach required the preparation of GTP-bound and GDP-bound Arf1, respectively, which was achieved by a standard method\(^\text{38}\). His-tagged human and \textit{P. falciparum} Arf1, minus the N-terminal 17 amino acids containing the myristoylation site and amphipathic α-helix (\textit{Hs}Arf1 and \textit{Pf}Arf1, respectively), were incubated with GTP or GDP in the presence of EDTA, followed by the addition of Mg\(^{2+}\) to stabilise the attached nucleotide. The \textit{Hs}Arf1 conformational change induced by GTP binding was monitored by kinetic and end-point intrinsic tryptophan fluorescence reads (Fig. 1a,b), as well as by performing native PAGE on the final protein preparations (Fig. 1c). As anticipated by the high level of sequence conservation in \textit{Pf}Arf1, the results confirmed that this is a viable approach for preparing and assessing GTP- and GDP-bound \textit{Hs}Arf1 and \textit{Pf}Arf1 (Fig. 1d–f), although the native PAGE mobility difference between \textit{Hs}Arf1-GTP and -GDP was smaller than observed with the human protein. The kinetic tryptophan fluorescence measurements further suggested that the original \textit{Hs}Arf1 preparation was purified from \textit{E. coli} as a mixture of GTP- and GDP-bound proteins (based on the respective increase and decrease in fluorescence during incubation with GTP and GDP), while \textit{Pf}Arf1 was predominantly GTP-bound.
To determine if GST-GGA3GAT could be used to detect Arf1 activation status using the plate-based colorimetric assay format described above, the nucleotide-loaded Arf1 proteins were incubated in a nickel-NTA coated 96-well plate, followed by sequential incubations with GST-GGA3GAT and a colorimetric GST substrate and absorbance readings performed at 340 nm (Fig. 1g). GTP- vs GDP-bound \( \text{N}^{\Delta 17}\text{HsArf1} \) could be robustly distinguished by the level of GST enzyme activity captured in the wells and the \( \text{N}^{\Delta 17}\text{PfArf1} \) results further confirmed that selective nucleotide-dependent GGA3GAT binding ability is conserved in the malaria protein. To confirm that selective binding of \( \text{N}^{\Delta 17}\text{PfArf1}-\text{GTP} \) to GST-GGA3GAT was due to the recognition of the GGA3GAT portion of the

Figure 1. Microtiter plate GST-GGA3GAT binding assay using GTP and GDP preloaded Arf1 proteins. (a,d) Five \( \mu \)M \( \text{N}^{\Delta 17}\text{HsArf1} \) (a) and \( \text{N}^{\Delta 17}\text{PfArf1} \) (d) were incubated at 25 °C with 50 \( \mu \)M GTP or GDP in the presence of 2 mM \( \text{N}^{\Delta 17}\text{HsArf1} \) or 20 mM \( \text{N}^{\Delta 17}\text{PfArf1} \) EDTA in a black 96-well plate and tryptophan fluorescence (Ex297/Em340) measured at 1 min intervals in a plate reader for 20 min. (b,e) After a further 40 min incubation, MgCl\(_2\) was added to a final concentration of 3 mM \( \text{N}^{\Delta 17}\text{HsArf1} \) or 30 mM \( \text{N}^{\Delta 17}\text{PfArf1} \), incubation continued for 10 min and \( \text{N}^{\Delta 17}\text{HsArf1} \) (b) and \( \text{N}^{\Delta 17}\text{PfArf1} \) (e) tryptophan fluorescence measured as an end-point reading. The nucleotide exchange reactions were conducted in triplicate wells and the data points represent mean fluorescence ± standard deviation. (c,f) After completion of nucleotide exchange, GTP and GDP loaded \( \text{N}^{\Delta 17}\text{HsArf1} \) (c) and \( \text{N}^{\Delta 17}\text{PfArf1} \) (f) were run on 12% native PAGE gels and stained with Coomassie. The gel images were cropped from two separate native PAGE gels, shown in Fig. S6 (Supporting Information). (g) GTP and GDP preloaded \( \text{N}^{\Delta 17}\text{HsArf1} \) and \( \text{N}^{\Delta 17}\text{PfArf1} \) were added to the wells of a Ni-NTA coated clear 96-well plate at a concentration of 1 \( \mu \)M and incubated for 30 min at 4 °C. An equal volume of GST-GGA3GAT was added to a final concentration of 1 \( \mu \)M and incubation continued for 60 min. After washing the wells, GST substrate solution containing reduced L-glutathione and 1-chloro-2,4-dinitrobenzene was added and absorbance measured at 340 nm after a 30 min incubation at room temperature. Mean background absorbance values obtained from empty wells (i.e. lacking immobilised Arf1) incubated with GST-GGA3GAT followed by GST substrate were subtracted from experimental readings. Incubations were carried out in triplicate wells and the bars represent mean Abs340 ± standard deviation. P-values were calculated by two-tailed t-tests.
Detection of ARNO-mediated nucleotide exchange by human and P. falciparum Arf1. To determine if the assay can be further exploited to detect the activation of Arf1 by an ArfGEF in vitro, GDP-loaded N17HsArf1 and N17PfArf1 were incubated with GTP in the presence of the Sec7 domain of ARNO (ARNOSec7) before adding the reactions to nickel-NTA coated plates and proceeding with the assay described above. ARNOsec7-mediated nucleotide exchange by both N17HsArf1 and N17PfArf1 could be discerned by a marked increase in GST-GGA3GAT binding compared to the respective controls (Fig. 2a,b). The controls consisted of the GDP-bound Arf1 proteins (N17HsArf1-GDP and N17PfArf1-GDP), the GDP-bound Arf1 proteins incubated with ARNOSec7 in the absence of GTP, and the GDP-bound Arf1 proteins incubated with GTP in the absence of ARNOSec7. To confirm that the enhanced GST-GGA3GAT binding was due to an increase in Arf1-GTP levels caused by ARNOSec7 stimulated nucleotide exchange, the reactions were repeated in the presence of 50 μM SecinH3, an inhibitor of the cytohesin family of ArfGEFs to which ARNO belongs. Inclusion of SecinH3 in the ARNOsec7 exchange reaction reduced GST-GGA3GAT binding by both N17HsArf1 and N17PfArf1 to levels obtained with control reactions lacking ARNOSec7 (Fig. 2c,d), causing a 93% and 74% inhibition of ARNOsec7-mediated N17HsArf1 and N17PfArf1 nucleotide exchange, respectively. The exchange reactions were subsequently repeated in the presence of 50 μM brefeldin A (BFA) or Golgicide A (GA), which are more selective for the BIG and GBF families of ArfGEFs as opposed to cytohesins. Consistent with this bias, neither compound inhibited ARNOsec7-mediated PfArf1 activation (Fig. 2f), while Golgicide A caused only a minor 26% inhibition of N17HsArf1 nucleotide exchange (Fig. 2e). In summary, the results confirmed that PArf1 is susceptible to Sec7-mediated nucleotide exchange in vitro. In addition, it suggested that the assay format can robustly detect the in vitro activation Arf1 by a Sec7 domain, as well as the specific inhibition of the reaction by small compound inhibitors.

Detection of GAP-mediated GTP hydrolysis by human and P. falciparum Arf1. Having demonstrated in vitro Sec7-mediated nucleotide exchange by PArf1, we next explored whether the assay format could detect GAP-mediated PArf1 deactivation, using the GAP domain of human ArfGAP1 (HsArfGAP1) as a model GAP. N17HsArf1 and N17PfArf1 preloaded with GTP were incubated with HsArfGAP1, added to a
murine malaria) parasites in genome-wide knockout and transposon mutagenesis studies. This led to the coding sequence has been reported to be essential to the survival of blood stage *P. falciparum*. *PfArfGAP2*, the homologue of *HsArfGAP1* since, in contrast to *PfArfGAP1* (PlasmoDB entry PF3D7_1244600) and *PfArfGAP2* (PF3D7_0526200.1). In contrast to the sequence conservation of *PfArfGAP1* given in Supplementary Information Fig. S3) and, while the crystal structure of the *PfArfGAP1* GAP domain has been published, neither GAP domain has been reported to have catalytic activity as well as its inhibition by a small molecule inhibitor.

**GAP activity of two putative *P. falciparum* ArfGAPs.** To some extent, stimulation of *PfArf1* nucleotide exchange and GTP hydrolysis by human Sec7 and GAP domains (as well as GTP-dependent binding to the human effector protein GGA3) was unexpected, given the high sequence and structural conservation of *PfArf1*. However, the question remains to what extent the predicted endogenous *P. falciparum* GEF and GAPs are capable of acting on *PfArf1*. In this study, we focused on the two sequences which are annotated as ArfGAPs on the plasmodb.org malaria genome database, which we designated as *PfArfGAP1* (PlasmoDB entry PF3D7_1244600) and *PfArfGAP2* (PF3D7_0526200.1). In contrast to the sequence conservation of *PfArf1*, the predicted amino acid sequences of the GAP domains of two proteins are considerably less conserved compared to human ArfGAPs (alignments with *HsArfGAP1* in Supplementary Information Fig. S3) and, while the crystal structure of the *PfArfGAP1* GAP domain has been published, neither GAP domain has been reported to have catalytic activity. To demonstrate the latter, we repeated the assays performed with *HsArfGAP1* GAP. GTP-loaded *PfArf1* was incubated with the GAP domains of the respective malarial ArfGAPs (*PfArfGAP1* GAP, *PfArfGAP2* GAP) and GST-GGA3GAT binding assessed (Fig. 3a,b). Controls included the GTP-loaded *PfArf1* proteins incubated in the absence of *HsArfGAP1* GAP and plate wells containing immobilised GDP-loaded *PfArf1* proteins. Incubation with the GAP domain completely abrogated the binding of GST-GGA3GAT to both *HsArf1* and *PfArf1*. To confirm that this was due to GAP-stimulated inactivation (GTP hydrolysis) of the *PfArf1* proteins, the ArfGAP inhibitor QS11 was included in the incubations of the GTP-loaded *PfArf1* proteins with *HsArfGAP1* GAP at a concentration of 50 μM, which preserved GST-GGA3GAT binding of both *HsArf1* and *PfArf1* (Fig. 3c,d). Collectively, the results confirmed that *PfArf1* is susceptible to GAP-mediated deactivation and that the assay format can competently detect *in vitro* ArfGAP activity as well as its inhibition by a small molecule inhibitor.

**Detection of GAP-mediated Arf1 deactivation using the GST-GGA3GAT binding assay.** (Figure 3.) The incubations of GTP-loaded *PfArf1* GAP were repeated in the presence of 50 μM QS11. Control reactions consisted of incubations of the GTP preloaded *PfArf1* proteins in the absence of *HsArfGAP1* GAP and QS11. Abs₃₄₀ values obtained from empty Ni-NTA plate wells incubated with GST-GGA3GAT were subtracted from all other readings. Incubations were carried out in triplicate wells and Abs₃₄₀ shown as mean ± standard deviation. P-values were calculated by two-tailed t-tests.

Identification of a selective small molecule inhibitor of *PfArfGAP1* GAP activity. To confirm that the reduction in GST-GGA3GAT binding when GTP preloaded *PfArf1* was incubated with 0.1 μM *PfArfGAP1* GAP and *PfArfGAP2* GAP was due to GAP activity, the assays were repeated in the presence of 50 μM QS11. In contrast to the results obtained with *HsArfGAP1* GAP (Fig. 3d), QS11 was unable to restore GST-GGA3GAT binding by *PfArf1*-GTP incubated with either *PfArfGAP1* GAP or *PfArfGAP2* GAP (Fig. 5a,b). To identify a potential inhibitor of *PfArfGAP1* GAP-mediated deactivation of *PfArf1*-GTP, we therefore screened a small BioFocus library of 1120 α-helix mimetics at a concentration of 50 μM (Screening details in Supplementary Information Fig. S5). We focused on the GAP domain of *PfArfGAP1* since, in contrast to *PfArfGAP2*, the coding sequence has been reported to be essential to the survival of blood stage *P. falciparum* and *P. berghei* (murine malaria) parasites in genome-wide knockout and transposon mutagenesis studies. This led to the
Identification of Chem1099 (Fig. 5c) which, at a concentration of 50 μM, preserved the GST-GGA3GAP binding ability of N\textsubscript{A17}P\textsubscript{Arf1}-GTP incubated with P\textsubscript{ArfGAP1}GAP, presumably due to inhibition of the GAP activity of the latter (Fig. 5c). Interestingly, the compound was inactive in a parallel screen carried out with P\textsubscript{Arf1} and H\textsubscript{s}ArfGAP1GAP (not shown). Indeed, at 50 μM, Chem1099 failed to inhibit the GAP activity of either H\textsubscript{s}ArfGAP1GAP or P\textsubscript{ArfGAP2}GAP on N\textsubscript{A17}P\textsubscript{Arf1}-GTP, suggesting GAP selectivity (Fig. 5c). The inhibitory activity of Chem1099 was further confirmed using an alternative assay format. As described earlier, tryptophan fluorescence measurements can be used to assess the conformation of N\textsubscript{A17}P\textsubscript{Arf1} which reflects its GTP- vs. GDP-bound status. Incubation of N\textsubscript{A17}P\textsubscript{Arf1}-GTP with P\textsubscript{ArfGAP1}GAP reduced its tryptophan fluorescence to levels obtained with a N\textsubscript{A17}P\textsubscript{Arf1}-GDP control, reflecting stimulation of GTP hydrolysis by the GAP domain (Fig. 5d). By contrast, incubation of 50 μM Chem1099 in the reaction maintained N\textsubscript{A17}P\textsubscript{Arf1}-GTP fluorescence levels, suggesting complete inhibition of P\textsubscript{ArfGAP1}GAP activity. Dose-dependent inhibition of P\textsubscript{ArfGAP1}GAP activity by Chem1099 was demonstrated by incubating N\textsubscript{A17}P\textsubscript{Arf1}-GTP and the GAP domain with serial dilutions of the compound followed by the GST-GGA3GAT binding assay and yielded an IC\textsubscript{50} value of 4.7 μM (Fig. 5e). To determine if Chem1099 possesses anti-parasitic activity, a dose-response assay was conducted against cultured P. falciparum (3D7) parasites and parasite viability assessed using a plasmodial lactate dehydrogenase assay, which yielded an IC\textsubscript{50} of 13.9 μM (Fig. 5f). In conclusion, the results suggest that P\textsubscript{ArfGAP1}GAP activity can be inhibited by small compounds in vitro, that inhibitory compounds can discriminate between the GAP domains used in this study and that the assay format can be used to identify GAP inhibitors in compound libraries.

**Discussion**

Given the rapid growth rate of the P. falciparum malaria parasite and its reliance on vesicular trafficking to secrete proteins to internal organelles (notably specialised secretory organelles required for erythrocyte invasion), trafficking of proteins to and from the host erythrocyte cytoplasm, as well as an extensive endocytosis of erythrocyte cytoplasm, it is intriguing that, in contrast to mammalian cells, its genome only encodes one predicted Sec7 domain protein (ArfGEF) and two ArfGAPs (according to plasmodb.org annotations) to potentially regulate Arf GTPase function which is central to trafficking in mammalian cells. This is further compounded by the complexity of the parasite life-cycle which, in addition to the blood stages responsible for malaria pathogenesis, includes male and female gametocyte transmission stages, several stages in the Anopheles mosquito vector and human liver stages\textsuperscript{46}. Moreover, although 6 sequences have been annotated as putative ADP-ribosylation factors, four may be Arf-like proteins as opposed to canonical Arf GTPases, one (Plasmodb accession number PF3D7_1034700) appears non-essential for blood-stage parasite survival\textsuperscript{39,40} and only P\textsubscript{Arf1} has been characterised\textsuperscript{25-27,31}. We have focused on P\textsubscript{Arf1} and found that it binds to the GAT domain of the human effector protein GGA3 in a nucleotide-dependent manner, which allows it to be characterised in vitro using the plate-based assay format developed with human Arf1 as a model and reported here, as well as potentially allowing an assessment of its activation status in parasites using pull-down assays\textsuperscript{37}.

Like its human counterpart, we confirmed that P\textsubscript{Arf1} is susceptible to GDP/GTP nucleotide exchange stimulated by a Sec7 domain. Having used a cytohesin domain for this purpose, we are currently exploring...
whether the nucleotide exchange activity extends to the predicted endogenous *P. falciparum* ArfGEF, despite the unusual secondary structure arrangement of its Sec7 domain. In addition, we confirmed that *Pf*Arf1 deactivation can be achieved *in vitro* using the model GAP domain of human ArfGAP1 and that the GAP domains of the two putative *P. falciparum* ArfGAPs have equivalent catalytic GAP activities (based on EC50 values obtained in the assay format used here). Interestingly, despite the *Pf*Arf1 GAP activity displayed by the GAP domain of *Pf*ArfGAP2 and its presence in the parasite blood stages, it has been reported as non-essential for blood-stage parasite survival, in contrast to *Pf*ArfGAP1, *Pf*Arf1 and the putative ArfGEF. Along with the co-localisation of *Pf*Arf1 with the Golgi marker GRASP and the BFA sensitivity of parasite secretion and Golgi structure, this may suggest that the latter trio of proteins form the regulatory network that mediates Arf GTPase-dependent trafficking of secretory proteins through the parasite Golgi apparatus. However, we recognise the caveat that we have performed the assays with truncated *Pf*Arf1 and *Pf*ArfGAP1 and that interaction in *vitro* does not necessarily

**Figure 5.** Selective inhibition of *Pf*ArfGAP1 GAP activity by a small molecule inhibitor. (a,b) One μM GTP preloaded *N*17 *Pf*Arf1 was incubated with 0.1 μM *Pf*ArfGAP1 GAP (a) or *Pf*ArfGAP2 GAP (b) for 30 min at 37 °C in the presence of 50 μM QS11, transferred to a Ni-NTA coated 96-well plate and incubation continued at 4 °C for 30 min. GST-GGA3 GAP was added to 1 μM and incubation at 4 °C continued for 60 min, followed by washing, incubation with GST substrate and absorbance readings at 340 nm. Control reactions consisted of GTP preloaded *N*17 *Pf*Arf1 incubated in the absence of the respective GAP domains, or with the GAP domains in the absence of QS11. Abs340 values obtained from empty Ni-NTA plate wells incubated with GST-GGA3 GAP were subtracted from all other readings. Incubations were carried out in triplicate wells and Abs340 ± standard deviation obtained from triplicate wells. (c) One μM GTP preloaded *N*17 *Pf*Arf1 was incubated respectively with 0.1 μM *Pf*ArfGAP1 GAP, *Pf*ArfGAP2 GAP or *Hs*ArfGAP1 GAP in the absence or presence of 50 μM Chem1099 and the GST-GGA3 GAP binding assay repeated as described above. Bars represent mean Abs340 ± standard deviation obtained from triplicate wells. The structure of Chem1099 is shown to the right. (d) Incubation of 1 μM *N*17 *Pf*Arf1-GTP with 0.1 μM *Pf*ArfGAP1 GAP in the presence and absence of 50 μM Chem1099 for 30 min at 37 °C was repeated in a black 96-well plate and tryptophan fluorescence (Ex297/Em340) measured as an end-point reading. Additional wells contained 1 μM *N*17 *Pf*Arf1-GDP without *Pf*ArfGAP1 GAP or without Chem1099. Bars represent mean fluorescence ± standard deviation obtained from triplicate wells. P-values were calculated by two-tailed t-tests. (e) The GST-GGA3 GAP binding assay with Chem1099 was repeated with three-fold serial dilutions (50 μM – 0.2 μM) of the compound added to the incubation of *N*17 *Pf*Arf1-GTP with *Pf*ArfGAP1 GAP in triplicate wells. Percentage inhibition of *Pf*ArfGAP1 GAP activity was calculated from the Abs340 readings obtained at the various compound concentrations relative to the mean Abs340 obtained with *N*17 *Pf*Arf1-GTP incubated with *Pf*ArfGAP1 GAP in the absence of Chem1099 (0%) and wells incubated with *N*17 *Pf*Arf1-GTP alone (100%). A dose-response curve was generated from the plot of mean percentage *Pf*ArfGAP1 GAP inhibition ± standard deviation vs. Log(Chem1099 concentration) and the IC50 value derived by non-linear regression analysis using GraphPad Prism. (f) The antiparasomal activity of Chem1099 was assessed by incubating *P. falciparum* (3D7) parasites with a serial dilution of Chem1099 in triplicate wells for 48 h and determining percentage parasite viability (relative to untreated controls) using a plasmoidal lactate dehydrogenase assay. The IC50 value was derived by non-linear regression analysis of the % parasite viability vs. Log(Chem1099 concentration) plot using GraphPad Prism.
translate into temporal and spatial co-recruitment and interaction on membrane surfaces in vivo. Potentially, this could be interrogated by parasite co-localisation experiments and assessing the effect of specific ArfGEF and ArfGAP1 inhibitors on PfArf1 activation status in parasites.

In addition to exploring the activity of PfArf1 regulatory proteins, the motivation for developing the assay described here was to establish an assay that can robustly detect the inhibition of the Arf1 activation/deactivation cycle and is amenable to screening compound libraries in a microtiter plate-based format. Conceptually, Arf function can be disrupted by inhibiting GTP binding, effector binding, GEF-mediated nucleotide exchange or GAP-mediated GTP hydrolysis. As opposed to inhibiting the binding of substrates/co-factors of traditional metabolic enzymes, protein-protein interactions are extremely challenging to interrupt with drug-like molecules. It is therefore encouraging that this has been achieved with Arf1 (as well as Arf6), with the application of developing potential anti-cancer agents in mind. The focus of these studies has been on inhibitors of GEF-mediated Arf1 activation, but also includes the discovery of the GAP inhibitors QS11 and its derivatives. To support inhibitor discovery, plate-based human Arf1 screening assays that have been reported include a FRET assay for GEF activity, a fluorescence polarisation assay for GAP activity, and an additional fluorescence polarisation aptamer displacement assay specific for cytohesins and used to identify SecinH3. Relevant to these efforts, we show that the assay format reported here can competently detect the in vitro inhibition of ARNO Sec7-mediated human and P. falciparum Arf1 activation by SecinH3, as opposed to BFA and Golgicide A, as well as inhibition of the deactivation of both proteins by human ArfGAP1 using QS11. In addition, in a preliminary screen of a limited α-helix mimetic library, we identified Chem1099 as a low micromolar in vitro inhibitor of PfPaf1 deactivation by the GAP domain of PfArfGAP1, further supporting the notion that ArfGAP activity can potentially be inhibited by small chemical compounds and, given the inactivity of Chem1099 against the GAP domains of HsArfGAP1 and PfArfGAP2, that this can be ascribed esssentially to PfArf1 and PfArfGAP1 in blood-stage parasites, it is encouraging that Chem1099 inhibits blood-stage P. falciparum, albeit with a moderate IC50 of 14 µM compared to the low nanomolar activities obtained with standard antimalarials. However, the assumption that parasite inhibition is due to GAP inhibition is a tenuous one in the absence of extensive mode of action or validation studies. Validation experiments could conceptually include an assessment of the effect of Chem1099 on parasite Golgi structure and function (e.g. through secretion assays), effect of Chem1099 on Arf1 activation status in parasites using pull-down assays on treated parasite lysates, and assessment of Chem1099 IC50 modulation in ArfGAP1 overexpressing or silenced transgenic parasite lines. We are currently expanding our screening of libraries for PfArfGAP1 inhibitors, coupled with biological assays to determine if this avenue of disrupting the PfArf1 activation cycle is detrimental to parasite viability.

Methods

Plasmid constructs and protein expression. For the E. coli expression of the GST-GGA3GAT fusion protein (GST fused to the GAP domain - amino acids 107–286 - of human GGA3), pGEX-4T-2/hGGA3(GAT) (Addgene plasmid #79436, donated by Kazuhiro Nakayama) was used. The other coding sequences were ligated into the Nhel/BamHI (Arf1 sequences) or Nhel/Xhol sites of pET-28a(+) for expression as His-tagged proteins. The coding sequence of human Arf1 minus the N-terminal 17 amino acids (N17-HsArf1) was PCR amplified from pARF1-CFP (Addgene plasmid #11381, donated by Joel Swanson) and the corresponding P. falciparum Arf1 sequence (N17-PfArf1) from the full length PfArf1 sequence (PlasmoDB ID PF3D7_1020900) codon-optimised for human expression, synthesised and cloned into pBluescript II by GenScript (Hong Kong). The sequences for the GAP domain of human ArfGAP1 (HsArfGAP1GAP; amino acids 1–140; NCBI sequence NP_060679.1), Sec7 domain of ARNO (ARNO Sec7; amino acids 51–253; NP_004219.3) and the putative GAP domain of PfArfGAP1 and PfArfGAP2 (PfArfGAP2GAP; amino acids 1–161; PF3D7_0526200.1) were codon optimised for E. coli expression and cloned into pET-28a by GenScript. The sequence encoding the putative GAP domain of P. falciparum ArfGAP1 (PfAfGAP1GAP; amino acids 1–161; PF3D7_1244600.1) was PCR amplified from P. falciparum strain 3D7 genomic DNA. T7 Express lysis E. coli (New England Biolabs) cultured in LB broth was used as expression host for all proteins. Expression was induced after bacterial density had reached OD600 0.5–0.8 with 1 mM IPTG for 3 hours at 37 °C. Bacteria harvested from the induced cultures were lysed by a freeze/thaw cycle, resuspension in buffer containing 2 mg/mL lysozyme and probe sonication. Proteins were purified from the soluble supernatants by nickel-NTA agarose (His-tagged proteins) or glutathione agarose (GST-GGA3GAT) affinity chromatography. Purified proteins were buffer exchanged into assay buffer (25 mM HEPES, 150 mM KCl, 1 mM MgCl2, 1 mM DTT, pH 7.4) using desalting columns and protein concentrations determined using Bradford protein assay. Glycerol was added to a final concentration of 40% (v/v) and the proteins stored at –20 °C until use. More details on protein expression and purification are given in the Supplementary Information (Fig. S2).

Nucleotide loading of Arf1 proteins. To preload N17-HsArf1 with GTP or GDP, the protein was diluted to a final concentration of 5 µM in assay buffer (25 mM HEPES, 150 mM KCl, 1 mM MgCl2, 1 mM DTT; pH 7.4) supplemented with 2 mM EDTA and 50 mM GTP or GDP and incubated at 25 °C for 60 minutes. MgCl2 was added to a final concentration of 3 mM and incubation continued for a further 10 min. Nucleotide loading of N17/PfArf1 was carried out in the same manner, except that 20 mM EDTA and 30 mM MgCl2 was used. To monitor nucleotide binding, intrinsic tryptophan fluorescence was measured at Ex330/Em490 in a Spectramax M3 plate reader (Molecular Devices). In addition, after completion of nucleotide loading, proteins were analysed in a gel shift (native PAGE) assay. Native PAGE was carried out with a 12% resolving gel and 4% stacking gel using normal SDS-PAGE conditions, except that SDS was omitted from all buffers and reducing agents were omitted from the sample buffer. After electrophoresis, the gel was stained with Coomassie Brilliant Blue.

Plate-based GST-GGA3GAT binding assay. His-tagged N17-HsArf1 or N17/PfArf1 preloaded with GTP or GDP were diluted to 1 µM in assay buffer supplemented with 1% (v/v) bovine serum albumin (BSA), transferred...
to a Ni-NTA HisSorb 96-well plate (Qiagen) (50 µL per well) and incubated at 4 °C for 30 min with gentle agitation. GST-GGA3ΔGAT in 50 µL assay buffer was added to a final concentration of 1 µM and incubation continued for an additional 60 min at 4 °C. The protein solutions were aspirated, the wells washed twice in assay buffer containing 0.1% (v/v) Tween-20 followed by four additional washes with assay buffer. GST assay buffer (2 mM reduced L-glutathione and 1 mM 1-chloro-2,4-dinitrobenzene in phosphate-buffered saline, pH 7.4), pre-equilibrated to room temperature, was added to each well (200 µL/well), the plate incubated at room temperature for 30 min and absorbance read at 340 nm in a Spectramax M3 plate reader. Background absorbance readings were obtained from triplicate wells incubated with GST-GGA3ΔGAT in the absence of immobilised Arf1 and the mean absorbance subtracted from the absorbance values of the experimental GST-GGA3ΔGAT wells. Plates were prepared for re-use by rinsing the plate wells in water followed by a 10 min incubation in stripping buffer (20 mM sodium phosphate, 500 mM NaCl, 50 mM EDTA, pH 7.4), an additional wash in water and a 10 min incubation in recharging solution (0.1 M NiSO₄). After a final rinse in water, the plates were used immediately.

ARNO-mediated nucleotide exchange and GAP-mediated GTP hydrolysis assays. For nucleotide exchange assays, 1 µM Nα17HsArf1 or Nα17PfArf1 preloaded with GDP was incubated with 0.2 µM ARNOSec7 and 50 µM GTP in assay buffer containing 1% BSA in round-bottom plates (50 µL per well) at 37 °C for 30 minutes with continuous agitation. The reactions were transferred to a Ni-NTA plate and the plate-based GST-GGA3ΔGAT binding assay continued as described above. Negative controls included reactions without ARNO, without GTP, or without either. GAP assays were carried out in the same manner, except that Arf1 proteins preloaded with GDP were used, ARNO was replaced with 0.1 µM of the relevant GAP domain (HsArfGAP1ΔGAP, PfArfGAP1ΔGAP, PfArfGAP2ΔGAP) and the addition of GTP was omitted. Negative controls consisted of reactions lacking the GAP domains. To assess the inhibition of nucleotide exchange or GTP hydrolysis, 10 mM stocks of brefeldin A (BFA; Sigma-Aldrich), Golgicide A (GA; Sigma-Aldrich), SecinH3 (Tocris Bioscience) and Q511 (Tocris Bioscience) were prepared in DMSO. The inhibitors were added to the reactions in the round-bottom plate wells to a final concentration of 50 µM [inhibitors were added to the Arf1 solutions immediately before adding ARNO (BFA, GA or SecinH3) or the GAP domains (Q511)]. A corresponding volume of DMSO was added to control reactions lacking the inhibitors (solvent vehicle controls). GAP titration experiments with Nα17PfArf1 were carried out as described above, except that incubations were carried out with 1 µM Nα17PfArf1-GTP and 3-fold serial dilutions (0.5–0.002 µM) of the GAP domains. For compound library screening, 50 µL assay buffer containing 1% BSA, 1 µM Nα17PfArf1-GTP and 0.1 µM PfArfGAP1ΔGAP was incubated in the presence of 50 µM of the test compounds in round-bottom plates for 30 minutes at 37 °C (compounds were added to the reaction mixture before the addition of the GAP domain). The reaction mixtures were transferred to Ni-NTA plates and the GST-GGA3ΔGAT binding assay continued as described above. Dose-dependent inhibition of PfArfGAP1ΔGAP by Chem1099 was determined in the same manner, using 3-fold serial dilutions of the compound. Percentage inhibition of GAP activity at the respective compound concentrations was calculated from Abs340 readings relative to those obtained in the same manner, using 3-fold serial dilutions of the compound. Absorbance readings were converted to percentage parasite viability relative to readings obtained from control wells (parasite cultures without Chem1099) and IC₅₀ derived by non-linear regression analysis of the resulting % viability vs. Log([Chem1099]) generated and the IC₅₀ determined using non-linear regression analysis with GraphPad Prism (v.8.2.0).

Antiplasmodial assay. This was carried out as described previously. Briefly, cultures of Plasmodium falciparum (3D7) parasites in a 96-well plate were incubated with a 3-fold serial dilution of Chem1099 (100–0.046 µM) for 48 h and parasite levels assessed using a colorimetric plasmoidal lactate dehydrogenase (pLDH) assay. Absorbance readings were converted to percentage parasite viability relative to readings obtained from control wells (parasite cultures without Chem1099) and IC₅₀ derived by non-linear regression analysis of the resulting % viability vs. Log(Chem1099) concentration using GraphPad Prism.

Data availability
The majority of the data generated or analysed during this study are included in this article and Supplementary Information. Data not shown are available by request from the corresponding author.

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References
1. D’Souza-Schorey, C. & Chavrier, P. ARF proteins: roles in membrane traffic and beyond. Nat. Rev. Mol. Cell. Biol. 7, 347–358 (2006).
2. Donaldson, J. G. & Jackson, C. L. Arf family G proteins and their regulators: roles in membrane transport, development and disease. Nat. Rev. Mol. Cell. Biol. 12, 362–375 (2011).
3. Jackson, C. L. & Bouvet, S. Arfs at a glance. J. Cell Sci. 187, 4103–4109 (2014).
4. Beck, R., Ravet, M., Wieland, F. T. & Cassel, D. The COPI system: molecular mechanisms and function. FEBS Lett. 583, 2701–2709 (2009).
5. Bonifacino, J. S. & Lippincott-Schwartz, J. Coat proteins: shaping membrane transport. Nat. Rev. Mol. Cell Biol. 4, 409–414 (2003).
6. Boulay, P.-J. et al. ARF1 controls proliferation of breast cancer cells by regulating the retinoblastoma protein. Oncogene 30, 3846–3861 (2011).
7. Casalou, C., Faustino, A. & Barral, D. C. Arf proteins in cancer cell migration. Small GTPases 7, 270–282 (2016).
8. Boulay, P.-L., Cotton, M., Melançon, P. & Claign, A. ADP-ribosylation factor 1 controls the activation of the Phosphatidylinositol 3-kinase pathway to regulate epithelial growth factor-dependent growth and migration of breast cancer cells. J. Biol. Chem. 283, 3642–3634 (2008).
9. Davis, J. E. et al. ARF1 promotes prostate tumorigenesis via targeting oncogenic MAPK signaling. Oncotarget 7, 39834–39845 (2016).
10. Prieto-Dominguez, N., Parnell, C. & Teng, Y. Drugging the small GTPase pathways in cancer treatment: promises and challenges. Cells 8, 255 (2019).
11. Ohashi, Y. et al. AMF-26, a novel inhibitor of the Golgi system, targeting ADP-ribosylation factor 1 (Arf1) with potential for cancer therapy. J. Biol. Chem. 287, 3885–3897 (2012).
12. Ohashi, Y. et al. M-COPA, a Golgi disruptor, inhibits cell surface expression of MET protein and exhibits antitumor activity against MET-addicted gastric cancers. Cancer Res. 76, 3895–3903 (2016).
13. Sausville, E. A. et al. Antiproliferative effect in vitro and antitumor activity in vivo of brefeldin A. Cancer J. Sci. Am. 2, 52–58 (1996).
14. Sztul, E. et al. ARF GTPases and their GEFs and GAPs: concepts and challenges. Mol. Biol. Cell 30, 1249–1271 (2019).
15. Spannhake, W., Shibata, Y. & Randazzo, P. A. ARF GTPases and kinesin of vesicle generation. FEBS Lett. 584, 2646–2651 (2010).
16. Seelaer, K. et al. Synthesis and biological properties of novel Brefeldin A analogues. J. Med. Chem. 56, 5872–5884 (2013).
17. Saenz, J. B. et al. Golgicide A reveals essential roles for GBF1 in Golgi assembly and function. Nat. Chem. Biol. 5, 157–165 (2009).
18. Vialud, J. et al. Structure-based discovery of an inhibitor of Arf activation by Sec7 domains through targeting protein-protein complexes. Proc. Natl. Acad. Sci. USA 104, 10370–10375 (2007).
19. Spomer, R. A. et al. The secretion inhibitor Exx2 perturbs trafficking of Shiga toxin between endosomes and the trans-Golgi network. Biochem. J. 414, 471–484 (2008).
20. Hafner, M. et al. Inhibition of cytohesin by SecinH3 leads to hepatic insulin resistance. Nature 444, 941–944 (2006).
21. Zhang, Q. et al. Small-molecule synergist of the Wnt/β-catenin signaling pathway. Proc. Natl. Acad. Sci. USA 104, 7444–7448 (2007).
22. Singh, M. et al. Structure-function relationship studies of Q51, a small molecule Wnt synergistic agonist. Bioorg. Med. Chem. Lett. 25, 4838–4842 (2015).
23. Truong, R. M., Francis, S. E., Chakrabarti, D. & Goldberg, D. E. Cloning and characterization of Plasmodium falciparum ADP-ribosylation factor and factor-like genes. Mol. Biochem. Parasitol. 84, 247–253 (1997).
24. Lee, F.-J. S. et al. Identification and characterization of an ADP-ribosylation factor in Plasmodium falciparum. Mol. Biochem. Parasitol. 87, 217–223 (1997).
25. Stafford, W. H., Stockley, R. W., Ludbrook, S. B. & Holder, A. A. Isolation, expression and characterization of the gene for an ADP-ribosylation factor from the human malaria parasite, Plasmodium falciparum. Euk. J. Biochem. 242, 104–113 (1996).
26. Leber, W. et al. A unique phosphatidylinositol 4-phosphate 5-kinase is activated by ADP-ribosylation factor of Plasmodium falciparum. Int. J. Parasitol. 39, 654–653 (2009).
27. Thavayogarajah, T. et al. Alternative protein secretion in the malaria parasite Plasmodium falciparum. PLoS One 10, e0125191 (2015).
28. Hayashi, M. et al. A homologue of N-ethylmaleimide-sensitive factor in the malaria parasite Plasmodium falciparum is exported and localized in vesicular structures in the cytoplasm of infected erythrocytes in the brefeldin A-sensitive pathway. J. Biol. Chem. 276, 15249–15255.
29. Ogun, S. A. & Holder, A. A. Plasmodium yoelii: brefeldin A-sensitive processing of proteins targeted to the rhoptries. Exp. Parasitol. 79, 270–278 (1994).
30. Crarry, J. L. & Haldar, K. Brefeldin A inhibits protein secretion and parasite maturation in the ring stage of Plasmodium falciparum. Mol. Biochem. Parasitol. 53, 185–192 (1992).
31. Wickham, M. E. et al. Trafficking and assembly of the cytosol–dense complex in Plasmodium falciparum-infected human erythrocytes. EMBO J. 20, 5636–5649 (2001).
32. Benton, J., Mattei, D. & Lingelbach, K. Brefeldin A inhibits transport of the glycophorin-binding protein from Plasmodium falciparum into the host erythrocyte. Biochem J. 300, 821–826 (1994).
33. Cook, W. J., Smith, C. D., Senkovich, O., Holder, A. A. & Chattopadhyay, D. Structure of Plasmodium falciparum ADP-ribosylation factor 1. Acta Cryst. F66, 1426–1431 (2010).
34. Baumgartner, E., Wiek, S., Paprotka, K., Zauner, S. & Lingelbach, K. A point mutation in an unusual Sec7 domain is linked to brefeldin A resistance in a Plasmodium falciparum line generated by drug selection. Mol. Microbiol. 41, 1151–1158 (2001).
35. Wiek, S., Cowman, A. F. & Lingelbach, K. Double cross-over gene replacement within the Sec7 domain of a GDP-GTP exchange factor from Plasmodium falciparum allows the generation of a transgenic brefeldin A-resistant parasite line. Mol. Biochem. Parasitol. 138, 51–55 (2004).
36. Cook, W. J., Senkovich, O. & Chattopadhyay, D. Structure of the catalytic domain of Plasmodium falciparum ARF GTPase-activating protein (ARFGAP). Acta Cryst. F67, 1339–1344 (2011).
37. Cohen, L. A. & Donaldson, J. G. Analysis of Arf GTP-binding protein function in cells. Curr. Protoc. Cell Biol. 48, 14.12.1–14.12.17 (2010).
38. Cox, R., Mason-Gamer, R. J., Jackson, C. L. & Segn, N. Phylogenetic analysis of Sec7-domain-containing Arf nucleotide exchangers. Mol. Biol. Cell 15, 1487–1505 (2004).
39. Zhang, M. et al. Uncovering the essential genes of the human malaria parasite Plasmodium falciparum by saturation mutagenesis. Science 360, eaap7848 (2018).
40. Bushell, E. et al. Functional profiling of a Plasmodium genome reveals an abundance of essential genes. Cell 170, 260–272 (2017).
41. DePonte, M. et al. Wherever I may roam: protein and membrane trafficking in the malaria parasite. Mol. Biochem. Parasitol. 186, 95–116 (2012).
42. Matthews, H., Duffy, C. W. & Merrick, C. J. Checks and balances: DNA replication and the cell cycle in Plasmodium. Parasit. Vectors 11, 216 (2018).
43. Otto, T. D. et al. New insights into the blood-stage transcriptome of Plasmodium falciparum using RNA-Seq. Mol. Microbiol. 76, 12–24 (2010).
44. Toenhake, C. G. et al. Chromatin accessibility-based characterization of the gene regulatory network underlying Plasmodium falciparum blood-stage development. Cell Host Microbe 23, 557–569 (2018).
45. Arkin, M. R., Tang, Y. & Wells, J. A. Small-molecule inhibitors of protein–protein interactions: Progressing towards the reality. Chem. Biol. 12, 1102–1114 (2014).
46. Raj, M., Bullock, B. N. & Arora, P. S. Plucking the high hanging fruit: a systematic approach for targeting protein–protein interactions. Bioorg. Med. Chem. 21, 4051–4057 (2013).
47. Yoo, J. et al. ARF6 is an actionable node that orchestrates oncogenic GNAQ signaling in uveal melanoma. Cancer Cell 29, 889–904 (2016).
48. Bill, A. et al. A homogeneous fluorescence resonance energy transfer system for monitoring the activation of a protein switch in real time. J. Am. Chem. Soc. 133, 8372–8379 (2011).
49. Sun, W., VanHooke, J. L., Sondek, J. & Zhang, Q. High-throughput fluorescence polarization assay for the enzymatic activity of GTPase-activating protein of ADP-ribosylation factor (ARFGAP). J. Biomol. Screen. 16, 718–723 (2011).
50. Le Manach, C. et al. Fast in vitro method to determine the speed of action and the stage-specificity of anti-malarials in Plasmodium falciparum. Malaria J. 12, 424 (2013).
51. Lunga, M. J. et al. Expanding the SAR of nontoxic antimalarial indolyl-3-ethanone ethers and thioethers. ChemMedChem 13, 1353–1362 (2018).
52. Makler, M. T. & Hinrichs, D. J. Measurement of the lactate dehydrogenase activity of Plasmodium falciparum as an assessment of parasitemia. Am. J. Trop. Med. Hyg. 48, 205–210 (1993).
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Author contributions
H.C.H. conceptualised the study and wrote the manuscript, with contributions from all the authors. T.S. performed the experiments, F.D.K., A.N. and D.L. contributed to developing the methodology and reagents used and performed additional experiments reported in the Supporting Information, C.G.L.V. assisted with evaluating the compound screening results and J.M.P., A.L.E. and H.C.H. directed the study.

Competing interests
The authors declare no competing interests.

Additional information
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